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Using *Bean pod mottle virus* (BPMV) as virus-induced gene silencing (VIGS) vector to target multiple genes in *Glycine max* (L.) Merr.

A dissertation

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Summary

Plant viral vectors are valuable tools for host gene analysis, thus possessing important applications as reverse genetics tools for gene function studies. *Bean pod mottle virus* (BPMV) is a bipartite, positive-sense (+) RNA virus of *Secoviridae*. Insertion of relatively short cDNA fragments (96nts) corresponding to different regions of soybean (*Glycine max*) phytoene desaturase1 (GmPDS1) into the 3' UTR of BPMV RNA2 initiated weak photobleaching manifested by white spots along the veins of soybean leaves. MFold algorithm was used to predict the RNA secondary structure of a 66 nucleotide region (nt #400-466) within the 5' UTR of BPMV RNA2. This region could potentially fold into a stem-loop (SL) designated as SLC. The functional analysis indicated the importance of the bottom section of the stem because disrupting this portion of structure completely abolished the BPMV infectivity. A 47 nt region, SLA, spanning from nt #263-309 of the 5' UTR of BPMV RNA2 was deleted. Nonviral inserts (GmPDS1 or GmPDS1/GmDCL4) of up to 325 and 625 nt, respectively, were tolerated by the same region and successfully down regulated the expression of their corresponding genes. Furthermore, one insertion mutant, V5UE-GmPDS1/GmDCL2, underwent recombination in the infected plants, leading to the truncation of 112nts (nt #250-361).

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List of abbreviations

Abbreviation	Full name
^0C	Degree Celsius
ACT	Actin
AGO	Argonaute
bp	Base pair
cDNA	Complementary DNA
CHS	Chalcon synthase
CP	Coat Protein
CPMR	Coat protein-mediated resistance
C-Pro	Co-factor protease
DCL	Dicer-like protein
DCR	Dicer
ddH_2O	Double distilled water
dpi	Day post inoculation
DRB	Double-stranded RNA binding protein
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EB	Ethidium bromide
EDTA	Ethylene di amine tetra acetate
eIF	Eukaryotic elongation factor
GFP	Green fluorescent protein
Gm	*Glycine max*
Hel	Helicase
Kb	Kilo base pair
Lb	Left border
L-CP	Large coat protein

MCS	Multiple cloning site
miRNA	MicroRNA
MIT	Massachusetts Institute of Technology
MP	Movement protein
nt	Nucleotide
ORF	Open reading frame
PAZ	Piwi, Argonaute, and Zwille
PCR	Poly chain reaction
PDR	Pathogen-derived resistance
PDS	Phytoene desaturase
PGR	Plant growth regulator
Post. ctrl.	Positive control
Pro	Protease
PTGS	Posttranscriptional gene silencing
Rb	Right border
RDM	RNA-mediated DNA methylation
RDR	RNA-dependent RNA polymerase
RISC	RNA induced silencing complex
RLC	RISC loading complex
RNAi	RNA interference
RT	Reverse transcriptase
SAM	*S*-adenosyl methionine
S-CP	Small coat protein
siRNA	Small interfering RNA
SL	Stem loop
SOB	Super optimal broth
ta-siRNA	*Trans*-acting RNA
TB	Terrific broth

T-DNA	Transfer DNA
UTR	Untranslated region
VIGS	Virus-induced gene silencing
VPg	Viral genome-linked protein
vsiRNA	Virus-derived siRNA
VSR	Viral suppressor of RNA silencing
WT	Wild type

Chapter One

Introduction
and
Literature Review

1.1 Introduction

In spite of being among the most damaging plant pathogens, plant viruses have been engineered to be used for heterologous protein expression, analysis of many viral functions, and virus-host interactions in intact plants. Furthermore, plant viral vectors offer applications as virus-induced gene silencing (VIGS) tools for reverse genetic studies of gene function. Vectors based on viruses have numerous advantages over conventional transgenic technology for protein expression and study of gene function. They are rapid, low cost, high-level expression of foreign gene, and can be used in variety of plant tissues (Burch-Smith *et al.*, 2004). However, these characteristics are hampered by limitations due to instability of, particularly large, foreign inserts, disruption of systemic virus spread after replacement of virus genes involved in movement.

Like other factors affecting the effectiveness of virus-induced gene silencing (VIGS), the relationship between the length of RNA sequence with a transgene and the ability to promote gene silencing in plants was investigated. The longer sequences inserted in the viral vector to initiate silencing, the more possibility to be lost during virus replication (Dolja *et al.*, 1993). Therefore, testing VIGS vector with inserts shorter than 100 nt-long for down regulation of a soybean gene needed to be investigated.

Distinct regions of (+) RNA viruses have been investigated for insertion or replacement with nonviral sequences, in which a foreign gene of interest replaced the capsid protein (CP) gene of a virus. Although these vectors could express a foreign gene, they did not have all the biological capabilities of the wild-type virus (Pogue *et al.*, 2002). Therefore, light has been shed on regions other than viral open reading frame (ORF) to develop plant virus-based vectors. The untranslated regions (UTRs) of positive-sense (+) RNA viruses at

both 5' and 3' termini of (+) RNA virus genomes commonly contain critical *cis*-acting elements required for genome replication and/or translation of virus-encoded proteins so that they are thought to be recalcitrant to large deletions or insertions. Indeed, the complete sequences of 5' and 3' UTRs are frequently retained in defective interfering (DI) RNAs, and must be included in minimal replicons of viruses (Wu *et al.*, 2009; Sztuba-Solinska *et al.*, 2013). In particular, the 5' UTRs of (+) virus genomes have been found to be extraordinarily sensitive to alterations of even a few nucleotides (nts), hence were rarely subjected to deletions or insertions of large sizes (Andino *et al.*, 1990; Niesters and Strauss, 1990; Filomatori *et al.*, 2006). Therefore, 5' UTRs of (+) RNA genomes were not known to tolerate large deletions (more than 100 nt) or nonviral insertions (more than 600 nt).

Therefore, the objectives of the study are the followings:

1. Shortening the time for virus inoculum production by introducing the viral cDNA-based constructs into lima bean cotyledons using particle bombardment.
2. Testing the feasibility of VIGS in soybean using *Bean pod mottle virus* BPMV-based vectors that contain short inserts.
3. Investigating the possibility of tolerating large deletions and/or insertions within the 5' UTR of BPMV RNA2.
4. Silencing multiple soybean genes simultaneously by insertion of their corresponding sequences into the 5' UTR of the same virus construct.

1.2 Literature Review

1.2.1 Overview of RNA silencing

During the last fifteen years, the view of eukaryotic gene regulation has been changed. The first hints of RNA silencing were observed in plants (Matzke and Matzke, 2004), fungi (Pickford *et al.*, 2002) and Nemattodes (Fire *et al.*, 1998).

Many plant researchers have attempted to maximize the production of proteins or enzymes by introducing extra copies of the gene of interest into the plant genome. In 1990, it has been published that overexpression of a gene coding for chalcon synthase (CHS), a key enzyme in anthocyanin biosynthetic pathway, to produce deep purple petunia flowers gave white and variegated flowers instead. This phenomenon, called "co-suppression", did not find a reasonable explanation at that time (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Further analyses showed that the expression of not only endogenous (CHS) gene but also introduced exogenous counterpart was decreased rather than increased. In other words, the transcription of both the endogenous and the introduced genes was normal, but the transcripts were degraded after transcription in sequence-specific manner. Thus, the level of protein expression was lower than that of wild-type plants (Jorgensen, 1990).

The molecular mechanism of RNA silencing has been established when Fire and colleagues described RNA silencing, named RNA interference (RNAi), in the nematodes by introducing long double strand (ds) RNA into *C. elegans* to trigger a sequence-specific degradation of corresponding mRNA (Fire *et al.*, 1998; Dykxhoorn and Leiberman, 2005). RNAi is present in eukaryotic cell under different names, such as post transcriptional gene silencing (PTGS) in plants (Hamilton and Baulcombe, 1999), quelling in fungi (Cogoni *et al.*, 1996) and algae (Wu-Scharf *et al.*, 2000).

1.3 Mechanism of RNA interference (RNAi)

The term RNA interference (RNAi) was coined to describe the down regulation of *Caenorhabditis elegans* genes using antisense or dsRNA (Fire *et al.*, 1998). Similar phenomenon was first observed in plant systems and has been referred to as post transcriptional gene silencing (PTGS) or co-suppression (Napoli *et al.* 1990; van der Krol *et al.*, 1990). The underlying mechanisms of these RNA-induced gene silencing processes are evolutionarily conserved in eukaryotic organisms ranging from yeasts to humans (Kurreck, 2009).

RNAi is a novel mechanism of gene regulation that limits the transcript level by either suppressing transcription [transcriptional gene silencing (TGS)] or by activating sequence-specific RNA degradation process [post transcriptional gene silencing (PTGS)]/RNA interference. As a result, RNAi or PTGS has evolved into generic term to describe most of these related mechanisms (Ding, 2000; Argawal *et al.*, 2003; Denli and Hannon, 2003).

In eukaryotic cells, RNAi is triggered by the intercellular presence of dsRNAs. The type III endoribonuclease (RNase III), called Dicer (DCR) in animals and Dicer-like (DCL) in plants (Jacobson *et al.*, 1999; Bernstein *et al.*, 2001), processes the dsRNAs into small RNAs of discrete sizes [(20-30 nucleotides (nt)] called small interfering RNA (siRNA) (Hamilton and Baulcombe, 1999).

siRNAs are RNA duplexes containing phosphate at 5' end and two unpaired nucleotides at 3' end of each strand (Zamore *et al.*, 2000). siRNAs are considered as the central component of RNA silencing system in all of the silencing systems that characterized to date. siRNAs are key mediators of RNAi process as they serve as sequence-specific guides to direct the cleavage of single-stranded RNAs with complementary sequence (Elbashir *et al.*, 2001;

Chen, 2010). After the processing of dsRNAs into siRNAs by Dicer, a series of cascades is triggered. siRNAs are incorporated into RNA-protein complex, the RNA induced silencing complex (RISC), where they guide a nuclease to the target mRNA (Hammond *et al.*, 2000). Each RISC contains one member of Argonaute (AGO) protein family at its catalytic core (Hutvagner and Simard, 2008; Vaucheret and Mallory, 2010). In most fungi and plants, host-encoded RNA-dependent RNA polymerases (RDRs) are also required for effective RNAi (Dalmay *et al.*, 2001; Ahliquist, 2002; Wassenegger and Krczal, 2006). Another family of dsRNA-binding domains called dsRNA-binding (DRB) proteins has been found to play a bridging role to associate with DCLs and/or AGOs (Kurihara *et al.*, 2006; Qu *et al.*, 2008; Jakubiec *et al.*, 2012).

1.4 Gene silencing components

1.4.1 Dicers

Dicers (DCRs) in animals or Dicers-like (DCLs) in plants and fungi are a family of dsRNA specific endonucleases (Bernstein *et al.*, 2001; Belvins *et al.*, 2006). Dicer family members are large multidomain proteins that contain putative RNA helicase, two tandem ribonucleases III (RNase III), and one or two dsRNA-binding domains. The tandem RNase III domains mediate the endonucleolytic cleavage of dsRNAs into siRNAs or micro RNAs (miRNAs), which mediate the regulation of mRNA expression (Hutvagner and Zamore; 2002; Tang *et al.*, 2002; Belvins *et al.*, 2006).

Several organisms have more than one copy of DCR gene and each one preferentially processes dsRNA that come from different sources (Meister and Tuschl, 2004; Belvins *et al.*, 2006). *Drosophila melanogaster* has two paralogs, Dicer-1 (DCR-1) which processes miRNA precursors in the cytoplasm and Dicer-2 (DCR-2) that is required for long dsRNA processing

(Liu *et al.*, 2003; Lee *et al.*, 2004). In *Arabidopsis thaliana*, four DCL enzymes (DCL1-4) have been identified (Park *et al.*, 2002; Belvins *et al.*, 2006).

DCL1 is required for miRNA precursors processing and two more proteins, HEN1 (S-adenosyl methionine-dependent methyltransferase) and HYL1 [dsRNA-binding (DRB) protein] are involved to do this process (Han *et al.*, 2004; Kurihara *et al.*, 2006). It has been reported that DCL2 and DCL4 contribute in processing of dsRNAs from virus origin into (22 and 21 nt) viral siRNAs, respectively (Deleris *et al.*, 2006; Qu *et al.*, 2008). In addition to its role against viruses, DCL3 is responsible for generating of 24 nt *cis*-acting siRNAs that derive from heterochromatin, transposons, and repeated elements and is involved in RNA-mediated DNA methylation (RDM) (Belvins *et al.*, 2006; Vaucheret, 2006).

1.4.2 Small RNA species

The machinery of RNAi is conserved among species. However, it has been found that RNAi pathways produce distinct classes of siRNAs duplexes. Several kinds of siRNA molecules such as siRNA (Hamilton and Baulcombe, 1999), miRNA (Lee *et al.*, 1993), *Trans*-acting RNA (ta-siRNA) (Yoshikawa *et al.*, 2005) have been identified.

1.4.3 Small interfering RNA (siRNA)

The discovery of siRNAs came to light when Hamilton and Baulcombe identified the product of RNA degradation as small dsRNA molecules, ca. 25nt, of both sense and antisense orientations (Hamilton and Baulcombe, 1999). siRNAs duplexes were first detected in plants undergoing either PTGS or virus-induced gene silencing and they were undetectable in control plants that were not silenced (Hamilton and Baulcombe, 1999). In animals, siRNAs were subsequently discovered in *Drosophila* tissue culture in which RNAi was

induced by introducing more than 500 nt long of exogenous RNA in *Drosophila* embryo extracts which carried out RNAi *in vitro* (Zamore *et al.*, 2000). siRNAs originate from transgenes (Hamilton and Baulcombe, 1999), endogenous repeat sequences, and transposons (Xie *et al.*, 2004; Bonnet *et al.*, 2006).

1.4.4 MicroRNA (miRNA)

miRNAs are 21-24 nt small RNA molecules that transcribed from non-coding RNA genes and have been identified to regulate variety of processes, such as development, metabolism, and stress responses (Bartel, 2004; Kim, 2005). The first discovered miRNA, *lin-4*, was described in 1993 when Ambros and colleagues (Lee *et al.*, 1993) screened the *C. elegans* mutants defective in the timing of post-embryonic development. It was found that *lin-4* locus produces a 22 nt non-coding RNA. The *lin-4* negatively regulates *lin-14*, which encodes a nuclear protein whose concentration must be reduced to allow worms to transit from the first larval stage to the second one. The negative regulation of *lin-14* by *lin-4* requires partial complementarity between *lin-4* and sites in the 3' untranslated region (UTR) of *lin-14*mRNA (Lee *et al.*, 1993).

More than 5000 miRNAs have been identified from various organisms, but most of them have not been functionally analyzed (Tang *et al.*, 2008). Like other RNA polymerase II transcripts, miRNAs are transcribed by RNA Pol II from miRNA genes, MIR, as primary miRNAs (pri-miRNAs) which range from hundreds to thousands of nucleotides in length (Fig. 1.1A). After transcription, pri-miRNAs comprising hairpin structure flanked by unpaired 5' and 3' ends are then capped and polyadenylated (Chen, 2005; Du and Zamore, 2005).

In animals, the maturation of miRNA involves two RNase III enzymes, Drosha, a nuclear RNase III and Dicer. The hairpin structure in pri-miRNA

molecule is processed by Drosha to yield hairpin with 2 nt 3' overhang called precursor miRNA (pre-miRNA) (Lee *et al.*, 2003; Chen, 2005; Kim, 2005). The resulting Drosha-processed pre-miRNAs which have 5' phosphate, 3' hydroxyl, and 2 nt 3' overhang are exported from nucleus to cytoplasm by protein called Exportin5 (Yi *et al.*, 2003; Bohnsack *et al.*, 2004). In the cytoplasm, the second RNase III enzyme (Dicer) processes the pre-miRNA into ca. 21nt mature miRNA duplexe, miRNA/miRNA*.

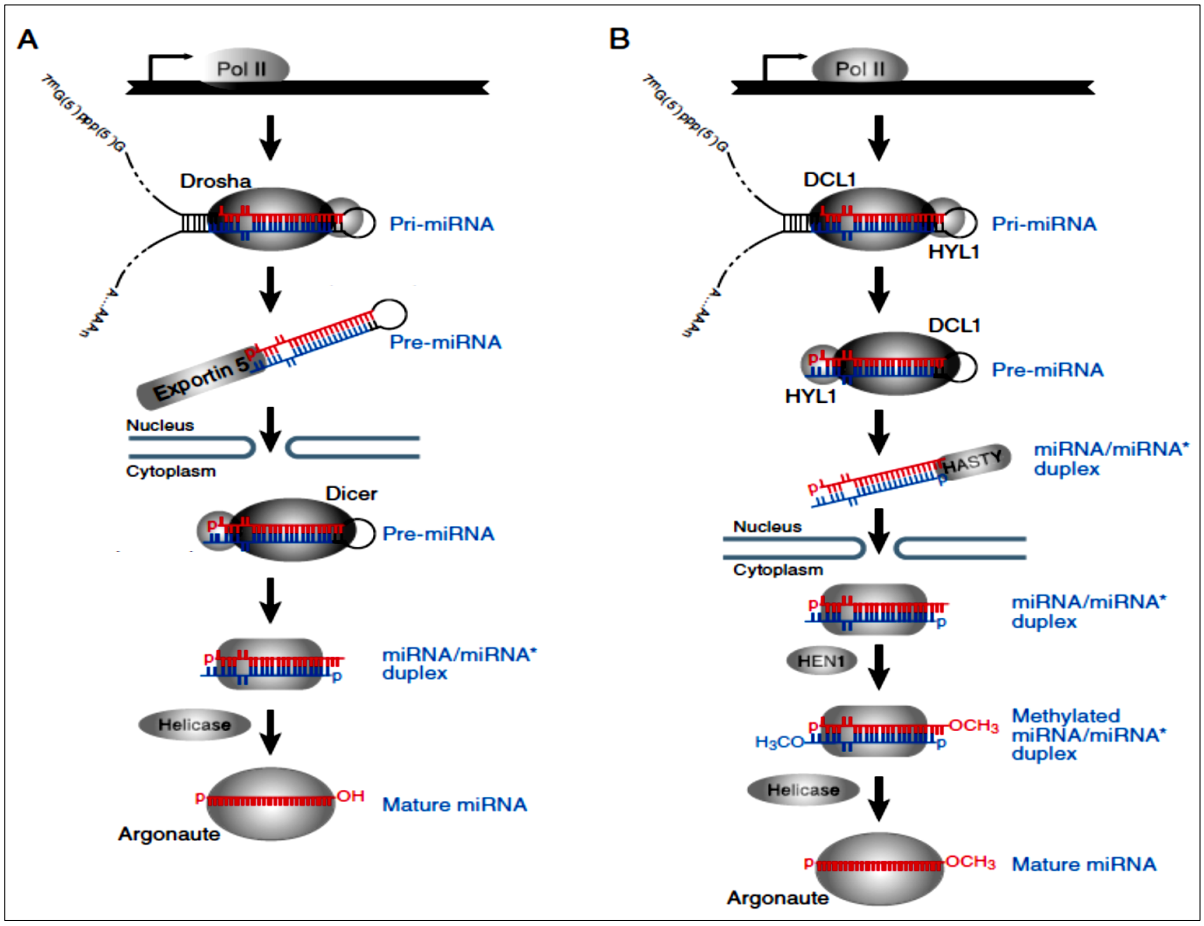

Figure 1.1: The miRNA biogenesis pathway. (A) Animal and (B) plant miRNA biogenesis. Mature miRNAs are red-colored strands, whereas the miRNAs* are in blue (Du and Zamore, 2005).

The resulting miRNA/miRNA* duplex has the same structure as the double stranded siRNAs but the former is partially complimentary to the miRNA*, the small RNA imperfectly matches with the miRNA within the pre-miRNA hairpin (Zamore *et al.*, 2000; Chen, 2005). The miRNA strand is

preferentially loaded into RISC complex to recruit the repression of gene expression, while the other strand, miRNA*, strand is degraded (Du and Zamore, 2005; Kim, 2005; Tang *et al.*, 2008).

Unlike animal-generated miRNAs, plant miRNAs are produced by DCL1 in both nucleus and cytoplasm since plants lack Drosha homolog (Fig. 1.1B) (Kurihara and Watanabe, 2004). While exportin 5 exports pre-miRNAs to the cytoplasm in animals, the plant ortholog exportin 5, HASTY (HST) exports miRNA/miRNA* duplex which is produced by DCL1 in the nucleus (Bollman *et al.*, 2003). Plant miRNAs is different from animal miRNAs which end with 2', 3' hydroxyl groups. Plant miRNA/miRNA* duplexes have methyl groups on the ribose of the last nucleotide of duplexes. These duplexes are methylated by *S*-adenosyl methionine (SAM)-dependent methyltransferase called HEN1 (Yu *et al.*, 2005).

There is an important distinction between siRNA and miRNA. siRNA originates by the cleavage of exogenous long stretches of perfectly base-paired nucleotides. On the other hand, double-stranded RNA with imperfect complementarity between the strands is cleaved to produce miRNA (Chen, 2010).

1.4.5 *Trans*-acting siRNA (ta-siRNA)

Trans-acting siRNAs (ta-siRNA) are plant-specific endogenous 21 nt small RNAs which are produced from non-protein coding genes called *TAS* genes such as *TAS1*, *2*, *3* and *4* (Yoshikawa *et al.*, 2005; Jouannet and Maizel, 2012; Xie *et al.*, 2012). ta-siRNAs have been identified as a result of analyses of small RNAs in *Arabidopsis thaliana* and they regulate their mRNA targets in *trans* (Vazquez *et al.*, 2004b). The generation of this kind of small RNAs requires all the factors involved in miRNA biogenesis. However, the precursors from which ta-siRNAs transcripts are initiated differ from that of miRNAs

(Allen *et al.*, 2005; Yoshikawa *et al.*, 2005). Furthermore, other components of PTGS pathway like RNA-dependent RNA polymerase (RDR6) and dsRNA-binding protein (SGS3) are implicated in ta-siRNAs biogenesis as shown in Fig. 1.2 (Howell *et al.*, 2007).

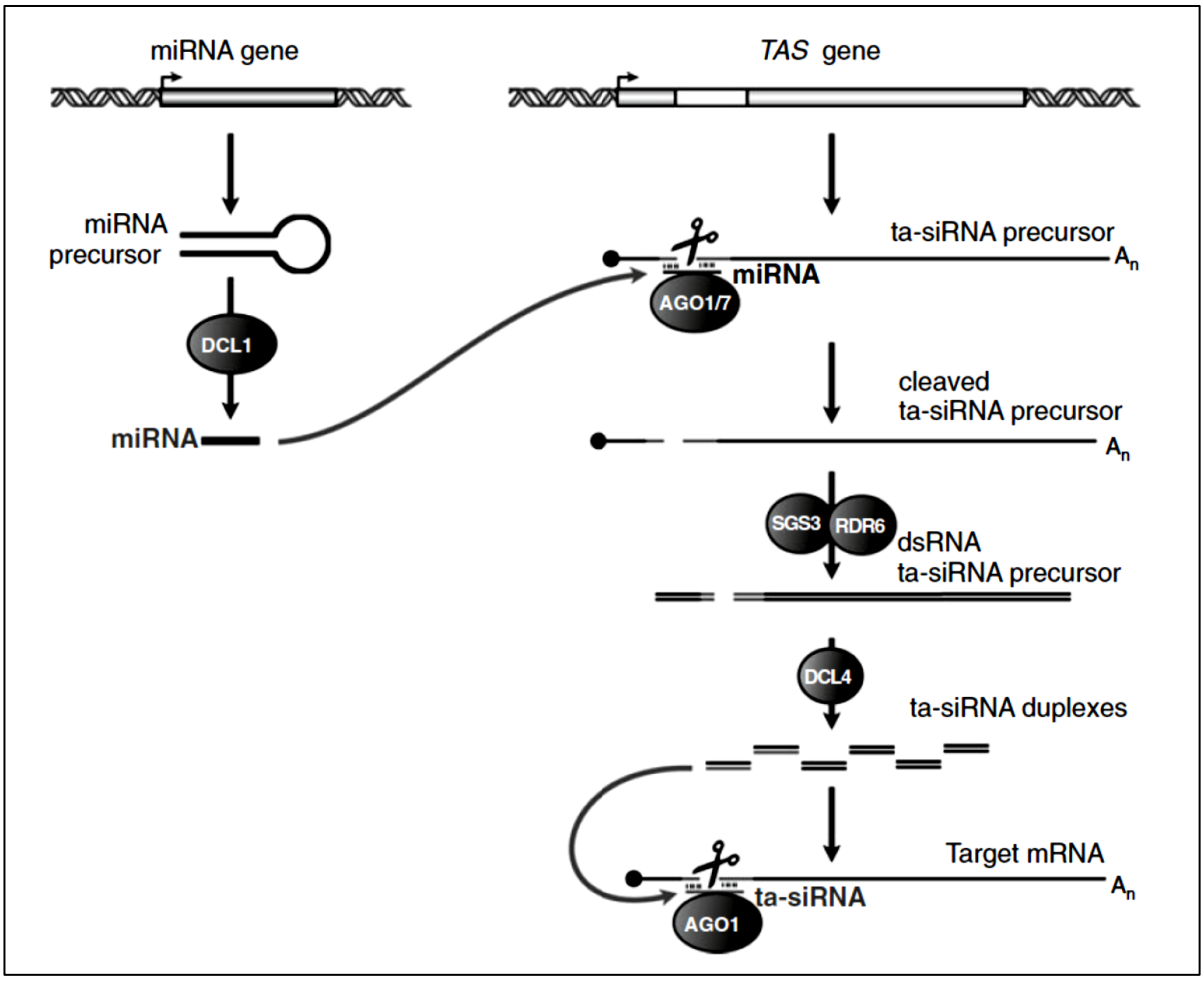

Figure 1.2: The ta-siRNAs biogenesis pathway. Ta-siRNA precursors mRNA are transcribed from TAS loci and cleaved by a miRNA loaded into AGO1 or 7. The processed ta-siRNA is converted to dsRNA molecule by RDR6 and SGS3, and then cleaved into ta-siRNA duplexes by DCL4. Once incorporated in AGO1, ta-siRNAs mediated cleavage of unrelated mRNA target (Jouannet and Maizel, 2012).

ta-siRNAs precursor transcripts arise from the transcription of one of the *TAS* genes by RNA pol II. Once transcribed, ta-siRNA precursors are cleaved by miR-mediated cleavage, i.e. AGO1/7 guided by miRNA cuts the transcribed ta-siRNA precursors. The resulting transcripts are then converted to double-stranded RNAs by the combinational action of RDR6 and SGS3. The dsRNAs produced are processed into 21 nt double-stranded ta-siRNAs by DCL4 (Allen

et al., 2005; Yoshikawa *et al.*, 2005; Howell *et al.*, 2007). ta-siRNAs are now loaded into RISC complex for targeting of mRNAs from other genes (Fig. 1.2).

1.4.6 Argonaute (AGO)

Besides the components of RNA silencing like DCLs, siRNAs and miRNAs that have been mentioned previously, it is prudent to shed the light on AGO proteins that represent the core component of RNAi machinery. Argonaute proteins (Tolia and Joshua-Tor, 2007; Hutvagner and Simard, 2008) were found to play an indispensable role in RNA silencing.

Screening of *Arabidopsis* mutants showing impaired development led to the discovery of AGO1, which is a member of so called AGO family (Benning, 1998). AGOs are small RNA binding proteins within RISC complex which guided by either siRNAs or miRNAs to target cleavage or translational suppression of complementary RNA (Hock and Meister, 2008).

AGO proteins are characterized by three functional domains, PAZ (Piwi, Argonaute and Zwille proteins), MID, and PIWI. The PAZ domain optimally binds the 3' overhangs of Dicer-generated small RNA duplexes. However, MID and PIWI domains consisting the C-terminal domain bind the 5' end of small RNA (Song *et al.*, 2004; Parker, 2010; Vaucheret and Mallory, 2010). Like the RNase H structure, it has been indicated that PIWI domain slices the RNA molecules (Parker *et al.*, 2005; Parker, 2010).

In eukaryotes, gene families encode diverse group of AGO proteins that expand the diversity of RNA silencing pathways that control the expression of endogenous genes, transgenes, and viruses (Vaucheret and Mallory, 2010; Harvey *et al.*, 2011). *Arabidopsis thaliana* has ten AGO paralogs which have distinct roles in different RNA silencing pathways (Hutvagner and Simard, 2008). For instance, among these ten AGO proteins, AGO1, 2, 5, and 7 have been implicated in antiviral RNA silencing (Qu *et al.*, 2008; Harvey *et al.*,

2011; Carbonell *et al.*, 2012). AGO1 and 7 have also been shown to execute the ta-siRNA-mediated gene silencing (Yoshikawa *et al.*, 2005).

1.4.7 RNA-dependent RNA polymerase (RDR)

RNA-dependent RNA Polymerase (RDR) was detected by screening for enzymes that catalyze the replication of plant RNA viruses (Fraenkel-Conrat, 1983). In plant, six RDRs (RDR1-RDR6) with different biological functions were characterized (Wassenegger and Krczal, 2006). RDR1 and RDR6 involve in antiviral defense in plants (Xie *et al.*, 2001; Schwach *et al.*, 2005; Qu *et al.*, 2005). RDR6-defecient *Arabidopsis* showed high susceptibility for *Cucumber mosaic virus* (CMV; Diaz-Pendon *et al.*, 2007) and viroids (Serio *et al.*, 2010). RDR6 involved not only in ta-siRNAs biogenesis (Howell *et al.*, 2007) but also in spreading of RNA silencing signals through the plasmodesmata and the phloem for long distance (Vaistij *et al.*, 2002; Himber *et al.*, 2003). For efficient silencing effect, RNA silencing is maintained by one of the RDRs which use the primary siRNA signals as templates to produce dsRNAs. RDRs-generated dsRNAs are then processed by one of the DCLs to secondary siRNAs which can move systemically to induce the silencing in cells that are distant from the region where the silencing is triggered. Thus, RDRs are required for maintenance but not for initiation of RNA silencing (Himber *et al.*, 2003; Tournier *et al.*, 2006).

1.5 RNA induced silencing complex (RISC) assembly

RISC assembly is the major complex in RNAi pathway. It requires sequential steps of RNA-protein and protein-protein interactions (Miyoshi *et al.*, 2009). Both siRNA and miRNA pathways are closely related in terms of biogenesis, assembly into RNA-protein complex and ability to regulate gene expression negatively in eukaryotes (Tang, 2005; Chen, 2010).

The DCR-generated siRNA or miRNA is double-stranded duplex. This duplex needs to be unwound prior to be incorporated into RISC complex. According to their thermodynamic stabilities at both ends, siRNAs are classified into two classes, symmetric siRNAs and asymmetric siRNAs (Schwarz *et al.*, 2003; Tang, 2005).

A symmetric siRNA is duplex with equally stable ends, thus both strands of siRNA are assembled into RISC with equal efficiency (Tang, 2005). On the other hand, an asymmetric siRNA has one end that is less stably paired than the other. Asymmetric siRNA is easily unwound from the less stable end. One strand of asymmetric siRNA is preferentially incorporated into RISC complex in a process called asymmetric assembly of RISCs (Schwarz *et al.*, 2003). Most miRNAs are asymmetric due to beginning of miRNA 5' ends with uracil that forms U:G wobble or mismatch base pairing that might facilitate RISC assembly (Du and Zamore, 2005).

The transition of DCR-cleaved siRNAs and miRNAs duplexes to single-stranded RNAs involves RNA-protein interaction called RISC Loading Complex (RLC) (Liu *et al.*, 2003; Tomari *et al.*, 2004; Liu *et al.*, 2006; Kim *et al.*, 2007; Noland and Doudna, 2013). RLC formation is the initial step of RISC assembly after the production of small RNAs. At this step, small RNAs are double stranded and ready to be unwound to produce "Guide strand" that is incorporated into the RISC and "Passenger strand" that is excluded and destroyed (Schwarz *et al.*, 2003; Matranga *et al.*, 2005).

In *Drosophila melanogaster*, Liu *et al.* (2003) reported that the components of RLC are siRNA duplex, DCR2 and R2D2 which contains two dsRNA-binding protein domains. Furthermore, Tomari *et al*. (2004) proved that R2D2 is important in sensing the asymmetry of siRNA duplex and it selects the strand that loaded into RISC. In other words, the orientation of

DCR2-R2D2 protein heterodimer determines which siRNA strand binds with the core RISC protein, AGO.

Active siRNAs contain 5'-phosphate groups on both ends (Elbashir *et al*., 2001). R2D2 preferentially binds the 5'-phosphate of the more stable end of passenger strand of siRNA duplex. However, DCR2 is found to bind the less stable end of guide strand (Tomari *et al*., 2004). Thus, D2R2 could serve as the sensor for the thermodynamic asymmetry of siRNA and orients DCR2 to the end that is easily unwound within the RLC. Liu *et al*. (2006) proved that neither DCR2 nor R2D2 alone can efficiently interact with the siRNA duplex in *Drosophila*. Interestingly, recent line of evidence indicated that mammalian DCR is unnecessary for asymmetric RISC assembly *in vitro* and *in vivo* (Betancur and Tomari, 2012).

Handing siRNAs from the RLC to the RISC requires an AGO, a core component of RISC complex. The RLC contains siRNA that is still unwound. Tomari *et al*. (2004) could not detect single-stranded siRNA in RLC assembled in *Drosophila ago2* mutants, which lack a functional AGO2. They concluded that unwinding of siRNA duplex occurs only when AGO2 is available. Thus, siRNA in RLC is unwound only when RISC is assembled.

Unwinding of siRNA and miRNA is still obscure. It has been hypothesized two mechanisms elucidating how strand separation of siRNA and RISC loading occur. These are done either by transferring the duplex to AGO then cleaving of the passenger strand by AGO in process called "passenger strand cleavage mechanism" or by RNA unwinding of the (ds) siRNA via an yet-unknown helicase followed by transfer of single-stranded guide strand to AGO "bypass mechanism" (Matranga *et al*., 2005). RNA silencing mechanism is well characterized in *Drosophila* to date. Matranga *et al*. (2005) postulated that siRNA-RISC assembly and maturation is done by AGO2 cleavage-assisted

mechanism of passenger strand. In contrast, for miRNA, passenger strand cleavage is unnecessary for incorporation of guide strand into RISC due to the presence of mismatch base pairings within the miRNA duplexes (Takeda *et al.*, 2008).

1.5.1 Mature RISC: target cleavage or translation repression

Mature or holo RISC comprises one kind of AGO protein which is tightly bound to the guide strand of either siRNA or miRNA (Song *et al.*, 2004; Parker, 2010). The RISC that contains siRNA called siRISC, however, miRISC has miRNA. siRISC and miRISC are functionally interchangeable and highly similar. Like miRISC, siRISC can repress translation of the target mRNA and miRISC can function as a siRISC to cleave the mRNA targets (Zeng *et al.*, 2003).

PIWI domain of AGO protein is similar to RNase H that cleaves the RNA strand of an RNA-DNA hybrid. AGO is called slicer if it has slicing activity that is attributed to the PIWI domain (Parker *et al.*, 2004; Parker *et al.*, 2005; Parker, 2010). The members of AGO family have been found to share conserved amino acid residues within the active site (Parker *et al.*, 2004). It has been thought that the AGOs with endonucleolytic activities are implicated in mRNA target cleavage. However, many AGOs that have conserved catalytic residues are endonucleolytically inactive. *A. thaliana* AGO4 which can function as a slicer has both catalytic and non-catalytic activities (Qi *et al.*, 2006).

The degree of complementarity between the siRNA and miRNA guide strands and their targets effectively determine the RISC-directed cleavage. Extensive pairing results in efficient cleavage. Furthermore, mismatches between the guide and the target are tolerated to certain level and RISC-directed cleavage could occur (Doench *et al.*, 2003; Saxena *et al.*, 2003).

Generally, the complementarity near to the 5' end of guide strand is more essential than that to 3' end of the guide. In *in vitro* study, Haley *et al*. (2003) reported that mismatches introduced into first 4-5 nt of the 5' end of the guide could be tolerated. By contrast, they showed that the mismatches created at the 3' end of the guide reached up to the last 9 nt of the 3' end.

RISC cleaves mRNA targets by hydrolyzing the phosphodiester linkage between the bases paired to nucleotides 10 and 11 of the guide RNA from the 5' end. Moreover, the target cleavage site is located between nucleotides 11 and 12 from the 3' end of the guide strand (Parker *et al.*, 2004; Song *et al.*, 2004).

In terms of gene silencing mechanism, perfect complementarity to target mRNA is characteristics of siRNA, so it causes degradation of target to which it is perfectly base paired. However, miRNA imperfectly base pairs to mRNA targets (Zeng *et al.*, 2003; Tang, 2005). The absence of extensive complementarity between the miRNA and the target directs the RISC to block the translation of the target rather than catalyzing its cleavage (Bartel, 2004; Brennecke *et al.*, 2005; Chen, 2005). siRNAs can function as miRNAs. By introducing number of mismatches to exogenous siRNAs, Doench *et al.* (2003) and Saxena *et al.* (2003) proved that mismatched siRNAs could efficiently silence the expression of endogenous genes by repressing translation.

In plants, most miRNAs have perfect or near perfect complementarity to their mRNA targets. Thus, plant miRNA-containing RISCs function as slicers to cleave the mRNA (Tang *et al.*, 2002; Bartel, 2004). In contrast to plant miRNAs, the level of complementarity between animal miRNAs and their targets is low. The binding of animal miRNAs is usually restricted to their 5' region (nucleotides 2-7 or 2-8) which is called seed region (Chiu and Rana, 2002; Du and Zamore, 2005; Kim, 2005).

Translational repression is enhanced by the binding of multiple small RNA-programmed RISCs to sites in the 3' UTR of the mRNA target (Xie *et al.*, 2005). The vast majority of animal miRNAs mediate translational repression and binding of miRNA seed region to the mRNA target is very crucial (Du and Zamore, 2005; Kim, 2005). Changes in base pairing between miRNA and its target at this region might greatly reduce the efficiency of target recognition or completely abolish the miRNA function. Weak base pairing at the seed region can be compensated by strong complementarity at the 3' end (Brennecke *et al.*, 2005). Since the seed region of a miRNA is so short (6-7 nt long), a single miRNA is predicted to approximately 100 target genes or target sites (Bartel, 2004; Ossowski *et al.*, 2008; Tang *et al.*, 2008).

Various mechanisms have been proposed to explain how small RNAs regulate gene expression, including translational repression, mRNA deadenylation, and decay (Pillia *et al.*, 2005; Wang *et al.*, 2008; Cooke *et al.*, 2010; Ricci *et al.*, 2012). Concerning translational repression, recent data strongly suggest that repression by miRNA takes place at the initiation step of translation (Pillia *et al.*, 2005; Bazzini *et al.*, 2012; Djuranovic *et al.*, 2012). The mechanism of repressing mRNA translation by miRNAs has been widely studied; however, there is still some controversy about the stage at which translation repression could be repressed.

Both 5' cap structure and poly (A) tail have been identified as essential factors for miRNA-mediated translational repression (Humphreys *et al.*, 2010; Watters *et al.*, 2010; Ricci *et al.*, 2012). In an *in vitro* study, Mathonnet *et al.* (2007) reported that inhibition of translation initiation is the earliest molecular event affected by miRNAs. The repression of translation initiation is possibly achieved by targeting the cap-binding eukaryotic elongation factor (eIF) 4F (Mathonnet *et al.*, 2007) and 4E (Zdanowicz *et al.*, 2009; Humphreys *et al.*, 2010).

Other observations suggested that full miRNA-mediated repression requires the poly (A) tail (Watters *et al.*, 2010; Ricci *et al.*, 2011). However, a study examining repression by miRNA in human cells documented that mRNA lacking a poly (A) tail could still be silenced (Beillharz *et al.*, 2009). Thus, miRNA-mediated translational repression is not affected by the presence of a poly (A) or its removal by deadenylation. To sum up, the precise molecular mechanism by which miRNAs mediate translational repression remains a matter of debate.

1.6 Few years before identifying of RNAi antiviral role

Prior to discovery of RNAi as a tool to combat viruses infecting plants, plant virologists sought to propose distinct strategies, such as pathogen-derived resistance (PDR), to produce virus-resistant plants. Coat protein-mediated resistance (CPMR) is known to be a kind of PDR (Beachy *et al.*, 1990; Scholthof *et al.*, 1993).

The first report documented the application of PDR against a plant virus was published in 1986 when Beachy and colleagues produced transgenic tobacco plants that expressed the coat protein (CP) gene of the tobamovirus *Tobacco mosaic virus* (TMV) (Abel *et al.*, 1986). Upon challenging with TMV particles, TMV CP-expressing transgenic plants either did not display symptoms of TMV infection or showed delayed symptoms after a period of time. Testing the concept of CPMR was successful to other plant viruses such as *Cucumber mosaic virus* (CMV) (Beachy *et al.*, 1990; Gielen *et al.*, 1996; Srivastava and Raj, 2008). From these findings, the emerging hypothesis was that CPMR, first, could be effective for many different virus groups. Second, increased levels CP transgene expression correlated with increased levels of resistance.

van der Vlugt *et al*. (1992) suggested that CP was not a molecule that confers resistance to the *Potato virus Y* (PVY) CP-expressing transgenic tobacco plants infected with PVY. They generated tobacco transgenic plants that expressed a non-translatable PVY CP gene, i.e. the transgenic plants produce non-translatable CP mRNA transcripts. Their observations were contrary to the results reported that the CPMR is due to CP itself. Surprisingly, the non-translatable PVY CP-expressing transgenic plants that inoculated with PVY did not show any symptoms of PYV infection. In addition, Lindbo and Dougherty (1992) reported that resistance of transgenic tobacco plants transformed with non-translatable *Tobacco etch virus* TEV-CP gene was RNA-mediated, not CP-mediated. Data concluded that the cytoplasmic RNA-mediated mechanism was responsible for virus resistance.

An unusual phenotype, which expanded the understanding of RNA-mediated virus resistance, was noticed during the work on the production of virus-resistant transgenic plants (Lindbo *et al.*, 1993). The term "recovery" is coined to describe the response of plant to the virus infection. In the recovery phenotype, transgenic plants that express a virus-derived transgene like CP initially become infected and the virus spreads systemically to the upper leaves that display the virus symptoms. However, by the time, the newly growing leaves look less symptomatic and subsequently each set of emerging leaves shows more asymptomatic appearance and virus free. This trend continues until leaves emerging from meristem no longer show symptoms of virus infection (Lindbo *et al.*, 1993; Jovel *et al.*, 2007). Furthermore, the virus-recovered plants that superinfected with either closely related virus or taxonomically distinct virus were completely susceptible and did not recover from the infection by other viruses (Ratcliff *et al.*, 1997).

Molecular analyses of the level of transgene-encoded RNA in asymptomatic leaf tissues in the recovered transgenic plants demonstrated that

nuclear transcription level of the transgene was normal, but there was tremendous reduction in the amount of transgene encoded RNA in the cytoplasm. Taken together, these findings suggested that virus resistance and low transgene mRNA level in the cytoplasm were due to sequence-specific posttranscriptional RNA degradation that functions in the cytoplasm (Lindbo *et al.*, 1993; Ratcliff *et al.*, 1999).

1.7 RNAi versus plant viruses

RNAi or PTGS, in plants, serves as a surveillance system in eukaryotic organism, and it detects various forms of dsRNA, initiating a cascade of events that degrade dsRNAs into siRNAs and miRNAs. One of the major roles of RNAi in plants is antiviral defense. RNAi eliminates the invading viral nucleic acid in sequence-specific manner (Waterhouse *et al.*, 2001; Qu, 2010; Pantaleo, 2011). In turn, viruses are able to disrupt RNAi at various steps by encoding suppressors that interfere with the RNAi components (Burgyan and Havelda, 2011; Lin *et al.*, 2012).

The first evidence supported the antiviral role of RNA silencing in plants was that viruses are potent inducers for RNA silencing in infected plants. This evidence was well illustrated by the detection of virus-specific siRNAs of both sense and antisense polarities in wild type plants infected with plus-strand RNA viruses (Hamilton and Baulcombe, 1999).

DCL-generated viral siRNAs were predicted to initiate from the dicing of the viral dsRNA replicative intermediate during the virus replication in the cytoplasm (Hui and HuiShan, 2012). As another source of viral siRNAs, highly structured regions like stem loop structures in some plant viruses have also been proposed to be potential substrates for DCLs (Du *et al.*, 2007; Mlotshwa *et al.*, 2008; Llave, 2010).

RNAi is a conserved mechanism among all eukaryotic organisms (Eamens *et al.*, 2008). Like other eukaryotes, plants use Dicer-like (DCL) and Argonaute (AGO) proteins as central enzymes of RNAi, which regulates gene expression and mediates defense against viruses (Ding *et al.*, 2004; Tolia and Joshua-Tor, 2007). In *A. thaliana*, 4 DCLs, 10 AGOs, 5 DRBs, and 6 RDR have been identified. They participate in different endogenous RNA silencing pathways to contribute in the regulation of gene expression throughout the plant life cycle (Park *et al.*, 2002; Xie *et al.*, 2004; Carbonell *et al.*, 2012). DCL1 and DCL4 process dsRNA to produce 21 nt long siRNAs, whereas DCL2 and DCL3 generate 22 and 24 nt long respectively (Takeda *et al.*, 2008).

All four DCLs were implicated in RNA-mediated antiviral defense. Many lines of evidence reported that DCL4 is primary producer of viral siRNAs (Deleris *et al.*, 2006; Du *et al.*, 2007; Wang *et al.*, 2011; Jakubiec *et al.*, 2012). DCL2 and DCL4 were identified to function in hierarchical manner (Deleris *et al.*, 2006). DCL4-generated 21-nt viral siRNAs are the most abundant species of viral siRNA in wild-type *Arabidopsis* plants infected with plus-strand RNA viruses. However, DCL2 alone can initiate RNA-based antiviral immunity in mutant plants lacking functional DCL4 (Bouche *et al.*, 2006; Deleris *et al.*, 2006). Furthermore, *DCL2* and *DCL4* double mutant plants have showed increased susceptibility to infection with different (+) RNA viruses (Deleris *et al.*, 2006). DCL3-derived viral siRNAs alone are insufficient to confer virus resistance, but they might enhance the antiviral defence mediated by DCL2 and DCL4.

Significant amounts of 24-nt long viral siRNAs that generated by DCL3 were detected in DNA geminivirus-infected plants, although 22 nt and 21 nt viral siRNAs produced by DCL2 and DCL4, respectively, have also been detected (Akbergenov *et al.*, 2006; Rodriguez-Negrete *et al.*, 2009). Low levels of all three species (21, 22, and 24 nt) viral siRNAs that target DNA virus,

Cauliflower mosaic virus, were observed in partial loss-of-function *DCL1*mutants. These observations indicated that DCL1 might facilitate the production of viral siRNAs by DCL2, DCL3, and DCL4 (Belvins *et al.*, 2006; Moissiard and Voinnet, 2006).

Several AGO proteins are implicated in antiviral RNA silencing, including AGO1, AGO2, AGO5, and AGO7. Qu *et al*. (2008) illustrated the participation of AGO1 and AGO7 against infection by mutated carmovirus *Turnip crinkle virus* (TCV) that does not express its silencing suppressor. Identification of virus-encoded suppressors that disrupt RNAi pathways illustrated the implication of certain AGOs in antiviral RNA silencing. Inhibition of AGO1 by viral suppressor, 2b, increases susceptibility to infection by (CMV) in *Arabidipsis* (Zhang *et al.*, 2006; Duan *et al.*, 2012).

It has been reported that AGO2 is also involved in antiviral defense against tombusvirus *Tomato bushy stunt virus* (TBSV) (Scholthof *et al.*, 2011), TCV and CMV (Harvey *et al.*, 2011). In the nucleus, AGO4 guided by DCL3-dependent-24nt siRNAs is required for transcriptional gene silencing via DNA methylation (Zilberman *et al.*, 2003; Havecker *et al.*, 2010). AGO4-deficient mutant plants are unable to maintain resistance to a DNA geminivirus *Beet curl top virus* (BCTV) mutant lacking the viral suppressor of RNA silencing protein, L2 (Raja *et al.*, 2008). This observation raised the possibility that viral siRNAs methylation is sufficient to confer resistance in plants against DNA viruses in the absence of any cleavage of viral transcripts (Raja *et al.*, 2008).

1.7.1 Amplification of viral siRNAs

The plant RNA silencing can be divided into two stages, initiation and maintenance (Ruiz *et al.*, 1998). The initiation stage depends on dsRNA trigger and siRNAs derived from that trigger. However, the maintenance stage is

independent of the dsRNA trigger and is responsible for the persistent silencing (Ruiz *et al.*, 1998; Vaucheret *et al.*, 2001; Vaucheret, 2006).

It has been shown that effective RNAi-based antiviral immunity in *A. thaliana* depends on the amplification of viral siRNAs that are initially processed from viral dsRNA replicative intermediate (Gracia-Ruiz *et al.*, 2010; Wang *et al.*, 2010). The RNA silencing is maintained by the action of a cellular RNA-dependent RNA polymerase (RDR), which synthesizes dsRNA by using the target mRNA as a template. The RDR-synthesized dsRNAs are then processed into "secondary siRNAs" by a DCL nuclease (Vaistij *et al.*, 2002; Wassenegger and Krczal, 2006).

The elevated RDR activity during virus infection roughly pointed to the implication of plant RDRs (Xie *et al.*, 2001). Among the six *Arabidopsis*-encoded RDRs, RDR1 and RDR6 participate in antiviral defense because mutants that defective for these proteins exhibited increased susceptibility to infection by some RNA viruses (Schwach *et al.*, 2005; Qu *et al.*, 2008; Wang *et al.*, 2010).

Production of virus-encoded suppressor proteins can mask the activity of RNA silencing factors like RDRs. Thus, wild-type plants look as virus-susceptible as that of mutant plants that lack the RNA silencing factors (Vaistij and Jones, 2009; Gracia-Ruiz *et al.*, 2010; Wang *et al.*, 2010). Diminishing expression of CMV-encoded suppressor protein, (CMVΔ2b), makes CMV non-pathogenic in wild-type and *RDR1*single *Arabidopsis* mutant plants. Therefore, production of CMV siRNAs is RDR1-dependent (Diaz-Pendon *et al.*, 2007). Other studies reported the role of RDR6 against virus invasion (Qu *et al.*, 2008; Wang *et al.*, 2010). Qu *et al.* (2008) demonstrated that *Nicotiana benthamiana* RDR6 was actively involved in defending tobacco plants from invasion by different RNA plant viruses, including members of the genera *Potexvirus*,

Carmovirus, and *Tobamovirus*. From these findings, it could be concluded that both RDR1 and RDR6 redundantly contribute in RNA-based antiviral defense (Qu *et al.*, 2008; Ding, 2010).

1.8 The mobility of silencing signal

The recovery phenotype, in which the uppermost leaves of infected plants become healthy and virus-free, is resistant to subsequent infection with the same virus (Lindbo *et al.*, 1993; Ratcliff *et al.*, 1997). The recovery phenomenon is a manifestation of spreading of RNA silencing. This effect can be explained through a movement of a mobile signal from the site of virus infection to distant tissues and can confer sequence-specific resistance (Molnar *et al.*, 2011).

Two kinds of silencing signal transmission have been identified, local transmission (short-distance) and systemic transmission (long-distance) (Voinnet *et al.*, 1998; Himber *et al.*, 2003; Ryabov *et al.*, 2004). In general, silencing signal was found to spread from the cells, into which the primary dsRNA-producing construct was introduced (Silencing inducer cells) into cells that do not undergo silencing yet (siRNA receiver cells) (Voinnet *et al.*, 1998; Himber *et al.*, 2003).

Agro-infiltration of GFP-expressing transgenic tobacco leaves with *Agrobacterium* culture carrying a GFP transgene construct abolished the expression of GFP in both infiltrated and non-infiltrated leaves (Himber *et al.*, 2003). They observed that GFP silencing occurred specifically in the infiltrated cells and moved over limited short distance (10-15 cells) into surrounding cell that non-infiltrated. RNA blot hybridization revealed that 21-nt long siRNAs were the most abundant in the silenced tissues. Therefore, it is proposed that 21-nt long siRNAs might be candidates to move over a limited and short distance (10-15 cells) via plasmodesmata into surrounding cells where they

mediate GFP-transcript cleavage (Voinnet *et al.*, 1998; Himber *et al.*, 2003; Ryabov *et al.*, 2004).

Contrary to the limited cell-to-cell movement, extensive cell-to-cell movement of silencing is characterized by spreading of silencing effect to a distance farther than 10-15 cells. Extensive silencing is mediated by transitivity of secondary siRNAs produced by the action of RDR6 (Voinnet *et al.*, 1998; Dalmay *et al.*, 2001; Vaistij *et al.*, 2002; Himber *et al.*, 2003). In transitivity, primary small RNAs, derived from the initial dsRNA target, induce the production of secondary small RNAs corresponding to regions outside the primary small RNA target site (Vaistij *et al.*, 2002; Himber *et al.*, 2003). The RDR6-synthesized dsRNAs serve as a source for secondary siRNAs by the dicing action of DCL, particularly DCL4 (Brosnan *et al.*, 2007; Moissiard *et al.*, 2007).

Transitive or secondary siRNAs move to 10-15 cells (siRNAs receiver cells) where the secondary siRNAs re-initiate a gene silencing. Thus, "siRNAs receiver cells" become "silencing inducer cells" and silencing effect extends into another set of 10-15 cells, leading to extensive cell-to-cell movement of silencing (Voinnet *et al.*, 1998; Himber *et al.*, 2003).

It has been proposed that extensive, but not limited cell-to-cell movement, was linked to transitivity mediated by RDR6 and SDE3, an RNA helicase required for dsRNA synthesis (Dalmay *et al.*, 2001; Himber *et al.*, 2003). Himber *et al.* (2003) designed an inverted repeat corresponding to the 5' region of GFP (IR-GF-FG). The IR-GF-FG fragment was inserted downstream the *Arabidopsis* sucrose transporter 2 (SUC2) promoter which is exclusively active in the phloem. Phloem-specific GFP silencing occurred and moved from the vascular tissue to the whole leaf when the SUC2-GF-FG construct was introduced into the GFP transgenic plants. In contrast, transformation of GFP

transgenic *RDR6* or *SDE3* mutants by SUC2-GF-FG resulted in a short-range movement of silencing from the phloem to the adjacent cells. Small RNA analysis indicated that the accumulation of 21-nt and 24-nt siRNAs corresponding to the 5' region (GF) in the *RDR6* and *SDE3* was similar to the transgenic plants having functional RDR6 and SDE3 (WT). Interestingly, 21-nt siRNAs specific to 3' region (P) of GFP were higher in WT than that of mutants. According to these results, Himber *et al.* (2003) strongly linked between the transitive 21-nt siRNAs synthesized by RDR6 and SDE3 and the extensive movement of silencing.

Systemic (long-distance) transmission of RNA silencing, in which the silencing signal is transmitted through the phloem (Voinnet *et al.*, 1998), was revealed by the grafting experiments (Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). Diminshing of nitrate reductase or nitrite reductas gene was manifested by the chlorosis of plant tissues. Silencing effect can be transmitted from the silenced rootstocks to the non-silenced transgenic scions by crossing the graft junction. Moreover, when a stem of WT plant was grafted between silenced transgenic rootstock and non-silenced transgenic scion, a silencing signal was transmitted through the non-transgenic WT stem (Palauqui *et al.*, 1997).

The effect of mobile RNA silencing is sequence-specific. This led to assume that RNA species is likely associated with the mobile silencing signal (Palauqui *et al.*, 1997; Himber *et al.*, 2003). Molnar *et al.* (2010) provided evidence that small RNAs of 22 and 24nt-long were able to cross the graft union, but the most abundant mobile silencing signal were 24-nt siRNAs. However, Dunoyer *et al.* (2010a) suggested that DCL4-dependent 21-nt siRNAs are necessary and sufficient for cell-to-cell and long-distance RNAi signaling. Taken together, multiple size classes of small RNA (21, 22, or 24-nt) have proposed to serve as mobile signals and contribute to induce RNA silencing in the recipient cells (Dunoyer *et al.*, 2010a; b; Molnar *et al.*, 2010).

1.9 Viruses can fight back

Even though RNA silencing-based defense against virus infection may still be an efficient barrier, there are many viruses are successfully able to infect plants. The discovery of the viral suppressors of RNA silencing (VSRs) provided additional clue to the RNA silencing as a tool against virus infection (Voinnet *et al.*, 1999; Burgyan, 2006; Burgyan and Havelda, 2011).

So far, none of the RNA silencing suppressors share any significant sequence homology with those from other viruses. This reflects that various VSRs are able to target the antiviral pathway at different stages, such as the viral RNA recognition, dicing (DCL step), RISC assembly, and the viral small RNA amplification as shown in Fig. 1.3 (Qu and Morris, 2005; Merai *et al.*, 2006; Zhang *et al.*, 2006; Burgyan and Havelda, 2011). Besides, their RNA silencing suppression activities, VSRs, perform multiple functions as coat proteins, movement proteins, helper components for the viral transmission, or as transcriptional regulators (Anandalakshmi *et al.*, 1998; Qu *et al.*, 2003; Bureau *et al.*, 2004).

Prior to their identification, VSRs have been known as viral proteins playing an important role in virus virulence (Pruss *et al.*, 1997; Brigneti *et al.*, 1998). The synergism response occurs when plants co-infected with two heterologous viruses show more severe disease symptoms than that of plants infected with each of the viruses alone (Pruss *et al.*, 1997). Members of potyvirus are characterized by expression of helper component-protease (HC-Pro) (Kasschau *et al.*, 1997). In plants infected with a potyvirus, the synergism was due to suppression of host defense mechanism by helper-component proteinase (HC-Pro). Moreover, it has been indicated that HC-Pro directly suppresses the antiviral RNA silencing mechanism (Anandalakshmi *et al.*, 1998, Brigneti *et al.*, 1998).

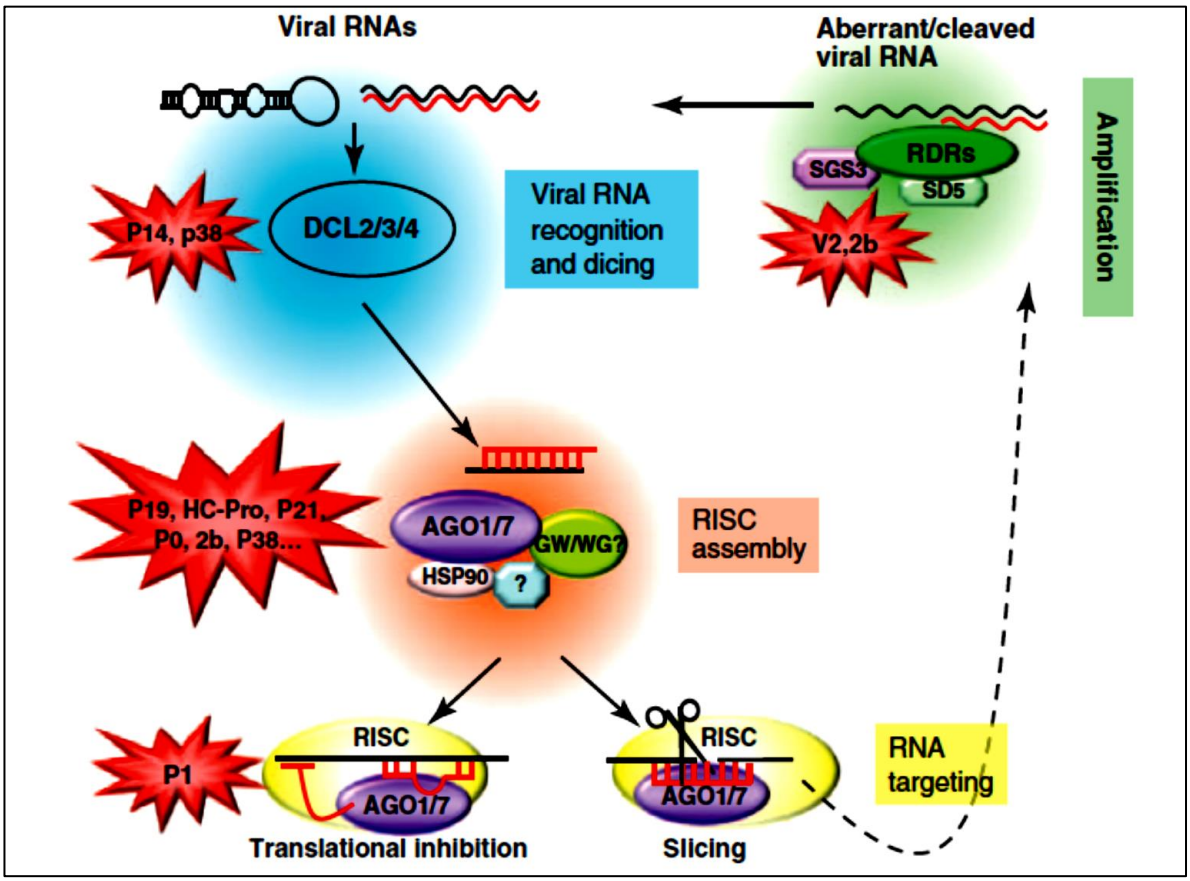

Figure 1.3: Suppression of various steps of RNA silencing-based antiviral defense by virus-encoded silencing suppressors. Viral dsRNAs or hairpin RNAs of viral origin are processed into vsiRNAs by DCLs (DCL2/3/4). vsiRNAs-loaded AGO1/7 interact with unidentified proteins (like GW/WG motifs containing proteins interacting with AGO) to assemble affective RISC. vsiRNA-loaded RISC targets viral RNAs slicing or translational arrest. Secondary vsiRNAs are produced via the actions of RDRs and their cofactors (SGS3 and SD5). Silencing suppressors can disrupt this pathway by preventing the assembly of different effectors or inhibiting their actions. (Burgyan and Havelda, 2011).

The inhibition of viral RNA recognition and dicing by plant Dicers is not a common strategy of the known VSRs. P14 of *Pothos latent aureusvirus* (Merai *et al.*, 2005) and P38 of TCV (Qu *et al.*, 2003) were identified to inhibit the processing of dsRNA to siRNAs. Moreover, P14 and P38 can bind dsRNA, inhibiting the DCL4 activity which is the prominent DCL in antiviral response (Qu *et al.*, 2003; Merai *et al.*, 2005). In addition to P14 and P38, the P6 VSR of CaMV has been shown to interfere with the processing of virus-derived siRNAs (Love *et al.*, 2007). Not only is P6 a viral suppressor of RNA silencing, but also it functions as a viral translational transactivator protein

essential for virus biology (Bureau *et al*., 2004). Haas *et al*. (2008) showed that P6 suppresses RNA silencing by interacting with dsRNA-binding protein 4 (DRB4), which is required for DCL4 function.

Many VSRs are able to block RISC assembly by targeting one of its essential components. The common feature of the most VSRs of different virus genera is siRNAs sequestration which prevents the assembly of the RISC effector (Fig. 1.3) (Lakatos *et al.*, 2006; Merai *et al.*, 2006; Ding and Voinnet, 2007). The P19 of tumbosviruses is the best known VSR so far. It specifically binds to 21-nt-long siRNAs, the products of DCL4 (Silhavy *et al.*, 2002). This result emphasized that P19 might suppress RNA silencing by sequestering siRNAs, thus preventing their incorporation into the RISC complex to serve as guides (Silhavy *et al.*, 2002; Lakatos *et al.*, 2004). VSRs that adopt siRNAs sequestration strategy specifically inhibit the initial step of the RISC complex formation. Lakatos *et al*. (2006) showed that none of the VSRs that have the ability to sequester siRNA blocks preassembled RISC activity *in vitro* or *in vivo*.

Different studies exhibited that various VSRs from distinct genera disrupt the RNA silencing through direct binding to AGO proteins. The 2b protein of CMV was the first protein identified that binds AGO1 *in vivo* (Zhang *et al.*, 2006). This protein also prevents the spread of a systemic silencing signal and facilitates the systemic virus infection (Guo and Ding, 2002). The 2b protein is co-localized with AGO1 and AGO4 in the cytoplasm and nucleus (Gonzalez *et al.*, 2010; Hamera *et al.*, 2012). It further inhibits the slicing activity of AGO1 by interacting with the PAZ domain and with part of the PIWI domain of AGO1 (Zhang *et al.*, 2006). Moreover, it is able to interfere and compete with AGO4 for binding to 24-nt siRNAs (Hamera *et al.*, 2012).

A new approach for binding AGO proteins is used by two distinct VSRs, P38 of TCV (Azevedo *et al.*, 2010) and P1 of *Sweet potato mild mottle virus* (SPMMV) (Giner *et al.*, 2010) see Fig. 1.3. These two VSRs mimic the cellular Glycine/Tryptophan (GW/WG) repetitive motif. The GW/WG motif was identified in different silencing-related proteins and it functions as "AGO hooks" to interact with Ago proteins (Karlowski *et al.*, 2010). Beside its ability to bind dsRNAs, the P38 protein has two GW motifs and interacts with *A. thaliana* AGO1 but not AGO4. Changing the GW to GA at the N terminal and C terminal of P38 abolished the interaction with AGO1. Point mutations in the P38 GW residues are sufficient to abolish TCV virulence which is restored in the *A. thaliana* AGO1 hypomorphic mutant. These findings revealed both physical and genetic interactions between the two proteins and suggested that P38 disrupts the sRNA loading into AGO1 by interacting with non-loaded AGO1 (Giner *et al.*, 2010).

Targeting virus-derived siRNAs (vsiRNA) amplification which requires the RDR6 activity by VSRs has been reported. The V2 protein encoded by *Tomato yellow leaf curl virus* (TYLCV) may interfere with the amplification of secondary vsiRNAs by interacting with suppressor of gene silencing-3 (SGS3) which interacts with RDR6 and involves in RDR6-mediated silencing amplification pathway (Glick *et al.*, 2008). SGS3 specifically binds dsRNA with a 5' ssRNA overhang (Fukunaga and Doudna, 2009). Glick *et al*. (2008) showed that V2 competes with the SGS3 protein for binding dsRNA with 5' ssRNA overhangs. However, V2 mutants that lack the suppression function *in vivo* cannot compete with SGS3 binding. This finding might also reveal a novel RNA intermediate formed during slicing, which is essential for SGS3/RDR6-dependent siRNA amplification in plants (Elkashef and Ding, 2009).

1.10 Virus-induced gene silencing (VIGS) vectors

The term "VIGS" was first reported by A. van Kammen to describe the phenomenon of recovery from virus infection (van Kammen, 1997). However, VIGS has been expanded to cover the technique involving recombinant viruses knocking down expression of endogenous genes (Ruiz *et al.*, 1998; Baulcombe, 1999). VIGS takes advantage of observations that viruses are potentially both initiators and targets of gene silencing. Early in the course of infection, the levels of viral genomic RNA and vsiRNAs could be relatively low and the viral RNA would be undetected by silencing. However, when a virus spreads systemically, more viral dsRNAs are produced and silencing could affect the viral RNA and the viral replication would slow down (Ratcliff *et al.*, 1997; Ruiz *et al.*, 1998).

Viruses are also able to silence host genes. In order to silence a gene of interest by VIGS, a recombinant virus vector (VIGS vector) is engineered to carry part of the desired gene sequence. Infection and systemic spreading of the recombinant virus cause specific silencing of the corresponding host gene within 1-3 weeks (Kumagai *et al.*, 1995; Ratcliff *et al.*, 2001).

Many plant viruses have been modified to produce VIGS vectors. *Tobacco mosaic virus* (TMV) was the first VIGS vector that developed to carry a sequence identical to *Nicotiana benthamiana* phytoene desaturase (Nb*PDS*) (Kumagai *et al.*, 1995). Transcripts of recombinant TMV carrying a sequence from the Nb*PDS* were produced and inoculated to *N. benthamiana* plants to successfully silence PDS (Kumagai *et al.*, 1995). A PVX-based VIGS vector was developed by Ruiz *et al.* (1998). Although this vector is more stable than the TMV-based vector, PVX has more limited host range than TMV. Furthermore, both TMV and PVX cause severe symptoms in their hosts, complicating interpretation of silenced phenotypes (Kumagai *et al.*, 1995; Ruiz

et al., 1998). *Tomato golden mosaic virus* (TGMV), a single-stranded DNA geminivirus, was used to silence distinct plant genes (Kjemtrup *et al.*, 1998). TMV, PVX and TGMV are unable to infect the meristems of host plants (Hull, 2004). Therefore, they are unlikely to be effective in silencing genes in those tissues. *Tobacco rattle virus* (TRV) was developed to overcome the limitations of host range and meristem exclusion (Ratcliff *et al.*, 2001; Liu *et al.*, 2002b).

A good VIGS vector should be able to infect the plant and spread systemically and uniformly, including the meristimatic tissues. Furthermore, it should not produce a strong silencing suppressor and infects a wide range of plants without causing severe symptoms that could interfere with interpretation of VIGS-induced phenotype (Lu *et al.*, 2003; Unver and Budak, 2009).

Structurally, VIGS vectors consist of three main parts: plasmid vector, full-length viral genome sequence, and the sequence corresponding to the host gene needed to be silenced. To construct an RNA plant virus-based VIGS vector, the virus RNA nucleotide sequence is converted to complementary DNA (cDNA) by reverse transcription and then inserted into an ordinary plasmid vector under the control of suitable promoter and terminator. This construct is called "infectious clone". Multiple cloning sites (MCS) can be introduced within the virus sequence for insertion of distinct sequences corresponding to the gene being silenced. Substituting the sequences that are dispensable for the virus infection with the foreign sequences is a common approach to produce VIGS vectors. Moreover, the foreign sequences could be inserted as an extra fragment within the virus vector (Kumagai *et al.*, 1995; Ratcliff *et al.*, 2001; Liu *et al.*, 2002b; Lu *et al.*, 2003). For instance, TRV is a plus single-stranded bipartite RNA virus (MacFarlane, 1999). The cDNA clones of TRV RNA1 and RNA2 were derived by CaMV 35S (35S) promoter and transcriptional terminator (T), then positioned between the right and left borders (RB and LB) of the T-DNA of plant binary transformation vector.

MCS site designed for gene silencing was introduced by replacing it with the non-essential gene in the RNA2 (Ratcliff *et al.*, 2001).

Preparation of RNA-based VIGS vectors inocula are performed either by propagation of the T-DNA –based virus construct in *Agrobacterium* (Grimsley *et al.*, 1986) or by *in vitro* transcription of viral cDNA (Kumagai *et al.*, 1995). Agroinoculation involves the use of Ti plasmid vector of *Agrobacterium tumefaciens* in which the T-DNA fragment is transferred to the genome of infected plant cells (Grimsley *et al.*, 1986). This process can be exploited if the full-length viral cDNA is present in the T-DNA and between a promoter and a transcriptional terminator that is active in plant cells (Ratcliff *et al.*, 2001; Liu *et al.*, 2002b). Infiltration of plant leaf by a syringe containing a suspension of the *Agrobacteria* is the most common method of agroinoculation used for VIGS (Vaghchhipawala and Mysore, 2008). However, *in vitro* transcription of viral cDNA is required to prepare infectious RNA viral transcripts *in vitro*. Rub inoculation of plant leaves with the viral RNA transcripts is then used to get viral infection (Kumagai *et al.*, 1995).

1.10.1 VIGS as a tool for functional genomics

In the past, forward genetics was the most reliable approach to identify gene function by isolating a mutant then cloning the mutated gene to identify the wild-type sequence responsible for such function (Baulcombe, 1999). Large-scale sequencing of *Arabidopsis* (reviewed by *Arabidopsis* Genome Initiative, 2000) and rice (*Oryza sativa*) (Goff *et al.*, 2002; Yu *et al.*, 2002) generates a large data base of sequences from several other plant species by homology-based sequence prediction. Despite identification of large sets genome sequences, function of these genes or DNA sequences, in many cases, needs to be analyzed (Yamada *et al.*, 2003; Haas *et al.*, 2005).

The availability of genome sequence has led to implement the approach of reverse genetics as an alternative to classical forward genetics. Reverse genetics investigates the function of a gene or DNA sequence by altering the expression of that sequence and identifying the mutated phenotype (Alonso and Ecker, 2006).

Silencing gene expression in a whole plant is one of the ways by which its biological function could be determined. Knocking out genes is the most frequent strategy used in reverse genetics to investigate gene functions (Reid *et al.*, 2009; Becker and Lange, 2010). It is achieved by insertion of transferred DNA (T-DNA) within the gene sequence to disrupt its function i.e. gene knocking out (Krysan *et al.*, 1999).

Virus-induced gene silencing (VIGS) is a novel powerful approach added to reverse genetics toolbox. VIGS functions via a posttranscriptional gene silencing (PTGS) mechanism by targeting and degrading RNA transcripts in a sequence-specific manner (Ratcliff *et al.*, 1997; Ruiz *et al.*, 1998). VIGS presents many advantages to circumvent the limitations of other functional genomics approaches like T-DNA-mediated mutagenesis. VIGS is rapid, cost effective, and easy. It does not require plant transformation and can be applied to plants which are recalcitrant to transformation (Constantin *et al.*, 2004; Diaz-Camino *et al.*, 2011). VIGS can be used to silence genes which their knocking out is embryo-lethal (Godge *et al.*, 2008; Senthil-Kumar and Mysore, 2011). The problem of functional redundancy due to the presence of gene families can be overcome by VIGS. Furthermore, it is possible to silence all or most members of a gene family by using a targeting sequence derived from the highly conserved region of a given family (Burch-Smith *et al.*, 2004; Burch-Smith *et al.*, 2006).

1.11 *Bean pod mottle virus* (BPMV)

1.11.1 Taxonomy and genomic properties

Bean pod mottle virus (BPMV) is a member of the secovirus genus in the *Comovirinae* subfamily, *Sercoviridae* family of order *Picornavirales* (Sanfacon *et al.*, 2009). Members of *Secoviridae* are characterized by having icosahedral particle structures, two or more different capsid protein subunits, and a very small viral genome-linked protein (VPg) which is covalently attached to the 5' of the virus genome. Moreover, many of secoviruses have bipartite RNA genomes (Le Gall *et al.*, 2008). BPMV has a bipartite positive single-stranded RNA genome consisting of RNA1 (approximately 6.0 Kb) and RNA2 (approximately 3.6 Kb) which are separately incapsidated in icosahedral particles of 28nm in diameter (MacFarlane *et al.*, 1991; Di *et al.*, 1999). The genomic RNAs have a small VPg linked to their 5' termini and are polyadenylated at the 3' end. BPMV RNAs encode one single polyprotein, from which multiple mature viral proteins are derived through posttranslational processing by the virus-encoded protease (Pro).

The RNA1-encoded polyprotein is the precursor for five proteins: a protease co-factor (C-Pro), a RNA helicase (HEL), VPg, Protease, (Pro), and the viral RNA-dependent RNA polymerase (RdRP). However, the RNA2 encodes a movement protein (MP) and two coat protein (CP) subunits, namely large CP (L-CP) and small CP (S-CP) (MacFarlane *et al.*, 1991; Di *et al.*, 1999; Le Gall *et al.*, 2005).

1.11.2 BPMV diversity, impact of infection and transmission

BPMV isolates were genetically classified into two distinct subgroups, I and II (Gu *et al.*, 2002). Their identification is based on nucleic acid hybridization of cloned cDNA probes that derived from RNA1 of BPMV

strains, Kentucky-Graves (K-G7) and Kentucky-Hancock (K-Ha1). The percentages of nucleotide sequence identity between strains K-G7, a prototype of subgroup I, and K-Ha1, a prototype of subgroup II, were 83.6% and 84.4% for RNA1 and RNA2 respectively (Gu *et al.*, 2002). Furthermore, Gu *et al*. (2002) isolated reassortant strains between the two subgroups I and II. These reassortants result from combining of RNA1 from subgroup I and RNA2 from subgroup II or vice versa (Gu *et al.*, 2002; Gu *et al.*, 2007).

Symptoms of plants infected with BPMV vary in severity. Severe symptoms are characterized by severe stunting, yellow mottling, leaf distortion, and extensive blistering, whereas yellow/green mottling with some stunting and blistering is characteristics of BPMV-induced moderate symptoms. However, mild green mottling and little or no stunting are the manifestations of the mild symptoms (Schwenk and Nickell, 1980; Gu *et al.*, 2002; Ziem *et al.*, 2007; Mozzoni *et al.*, 2009).

BPMV may also affect the appearance of soybean seeds. BPMV-infected soybean plants may produce seeds with mottled seed coats. Seed mottling originates at the hilum of seed and is also referred as "hilum bleeding", since buff, brown, or black color spreads from hilum to cover the whole seed. Seed mottling reduces seed quality and customers' acceptance (Giesler *et al.*, 2002; Hobbs *et al.*, 2003).

It has been found that BPMV strains of subgroup I and II induce moderate and mild symptoms, respectively regardless of soybean cultivar. However, the reassortant BPMV strains induce severe symptoms (Gu *et al.*, 2002; Zhang *et al.*, 2007). Symptom severity is associated with RNA1, not RNA2 (Gu *et al.*, 2007). Furthermore, C-Pro and C-terminal of HEL encoded by RNA1 are determinants of symptom severity (Gu and Ghabrial, 2005). Amino acid residues at positions 359 and 408 of C-terminus of HEL are responsible for

symptom severity. The changes from Serine (Ser) to Asparagine (Asn) at amino acid position 359 and from Leucine (Leu) to Phenylalanine (Phe) at amino acid position 408 differentiate between mild and severe strains (Gu and Ghabrial, 2005). BPMV-IA-Di1 isolate induces mild symptoms that indistinguishable from the uninfected mock control (Zhang *et al.*, 2010). Changing the amino acid residues which are both (Ser) at positions 359 and 356 to (Asn) at both positions enhanced symptom severity caused by BPMV-IA-Di1 (Zhang *et al.*, 2010).

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipment

The following apparatus and equipment were used throughout the experimental work:

Apparatus	Company, Origin
Analytical balance	Sartorius, Germany
Autoclave	Steris, USA
Centrifuge	Eppendorf, Germany
Cooled centrifuge	Thermo scientific, USA
Distiller	Thermo scientific
Gel electrophoresis system	Bio-Rad, USA
Gyratory shaker	Thermo scientific
Hot plate with magnetic stirrer	Thermo scientific
Laminar air flow cabinet	Labconco, USA
Microbalance	Sartorius
Micropipette	Gilson, France
MZFLIII dissecting microscope	Leica, Switzerland
NanoDrop	Thermo scientific
pH meter	Thermo scientific
Plant growth chamber	Conviron, Canada
Shaking incubator	New Brunswick scientific, USA
Thermocycler	Bio-Rad
Ultra low freezer	So-low, USA
UV transilluminator	UVP, USA
Vortex	Genie, USA
Water bath	Thermo scientific

2.1.2 Chemicals

Chemicals	Company, Origin
Agarose	Thermo scientific, USA
Ammonium nitrate	Fisher scientific, USA
Ampicillin sodium salt	Lab scientific, USA
Boric acid	Fisher scientific
Bromophenol blue	Fisher scientific
Calcium chloride hydrate	Fisher scientific
Celite® 545 AW	Sigma-Aldrich, USA
Chloroform	Fisher scientific
Cobalt chloride hydrate	Fisher scientific
Cupric sulfate hydrate	Fisher scientific
Ethanol	Fisher scientific
Ethidium bromide	Fisher scientific
Ethylene di amine tetra acetate (EDTA)	Fisher scientific
Ferrous sulfate hydrate	Fisher scientific,
Gelrite	Fisher scientific
Glacial acetic acid	Fisher scientific
Glycerol	Fisher scientific
Isopropanol	Fisher scientific
Magnesium sulfate hydrate	Fisher scientific
Manganese sulfate hydrate	Fisher scientific
myo-Inositol	Fisher scientific
Nicotinic acid	Fisher scientific
Potassium chloride	Fisher scientific
Potassium hydroxide	Fisher scientific
Potassium iodide	Fisher scientific
Potassium nitrate	Fisher scientific

Chemicals	Company, Origin
Potassium phosphate monobasic	Fisher scientific, USA
Pyridoxine. HCL	Fisher scientific
Sodium chloride	Fisher scientific
Sodium hydroxide	Fisher scientific
Sodium molybdate	Fisher scientific
Sodium phosphate dibasic	Fisher scientific
Sodium phosphate monobasic	Fisher scientific
Spermidine	Sigma-Aldrich, USA
Sucrose	Fisher scientific
Thiamine. HCL	Fisher scientific
Tris Base	Fisher scientific
Trypton	Fisher scientific
Xylene cyanol FF	Fisher scientific
Yeast extract	Fisher scientific
Zinc sulfate hydrate	Fisher scientific

Table 1. Primers for BPMV vectors modification, sequencing, foreign gene cloning and clones screening

Primer Name	Primer Sequence (5'→3')
BPMVII-2937F	GGAATCAAATGGCAACAGTGAGAACAC
BPMVII-1F (*Xho*I)	GAAGAACTCGAGTATTAAAATTTTCATAAGATTTGAAATTTTGA
BRIIG-904R (*Sal*I)	GAAGAAGTCGACTTAGTAGAATACAACTTGTCAAC
BPMV-V5UE-F	CAGATTTAAACTGGATCCTTAATTGGTACCACGTGAAATCTTGGATTAG
BPMV-V5UE-R	GATTTCACGTGGTACCAATTAAGGATCCAGTTTAAATCTGACACGAAGT
P35S-F2	CACAATCCCACTATCCTTCGCAAGA
BR2G-460*Bam*HI-F	GAAAAACACTTGGGCGTTGGATCCGTGCAAATGTTTGCTTCGTTA

BR2G-460*Bam*HI-R	TAACGAAGCAAACATTTGCACGGATCCAACGCCCAA GTGTTTTTC
GmDCL2-siRNA	GTCCCATTTGGATTGGTAAATTC
GmDCL4-siRNA	GAGCTGGACGAATACTTAATTTA
GmPDS1a-2052F	GATC GAAAGGCTCAGCTTCTGAAATTTAGCT
GmPDS1a-2078R	GATC AGCTAAATTTCAGAAGCTGAGCCTTTC
GmPDS1b-1353F	GATC ATAACTGGAAAGGGATTCCATATTTCC
GmPDS1b-1379R	GATC GGAAATATGGAATCCCTTTCCAGTTAT
GmPDS1c-1529F	GATC TATAGCCCAAACCAGTCAATGTTAGAT
GmPDS1c-1555R	GATC ATCTAACATTGACTGGTTTGGGCTATA
GmPDS1-1499F	GCTGACATGTCAGTAACTTGCAA
GmPDS1-1684F	CAAGTACCATGTTGTTAAAACACCA
GmPDS1-540F	GATTGGCTGGTTTATCAACTGCAA
GmPDS1-234F	GCTATATATCTGCTGCCAACTTCA
GmPDS1A1-1494F(96)	GATCTATATGCTGACATGTCAGTAACTTGCAAGGAAT ATTATAGCCCAAACCAGTCAATGTTAGAGTTGGTTTT TGCACCAGCCGAAGAATGGATTTCAC
GmPDS1A1-1589R(96)	GATCGTGAAATCCATTCTTCGGCTGGTGCAAAAACCA ACTCTAACATTGACTGGTTTGGGCTATAATATTCCTT GCAAGTTACTGACATGTCAGCATATA
GmPDS1B-1683F(96)	GATCTCAAGTACCATGTTGTTAAAACACCAAGGTCG GTTTACAAAACTGTTCCAAATTGTGAACCTTGTCGAC CCATTCAAAGATCTCCTATAGAAGGTT
GmPDS1B1-1778R(96)	GATCAACCTTCTATAGGAGATCTTTGAATGGGTCGAC AAGGTTCACAATTTGGAACAGTTTTGTAAACCGACCT TGGTGTTTTAACAACATGGTACTTGA
GmPDS1C1-539F(96)	GATCGGATTGGCTGGTTTATCAACTGCAAAATATTTG GCTGATGCTGGGCATAAACCTATATTGCTGGAAGCA AGAGACGTTCTAGGTGGAAAGGTTGCT
GmPDS1C1-634R(96)	GATCAGCAACCTTTCCACCTAGAACGTCTCTTGCTTC CAGCAATATAGGTTTATGCCCAGCATCAGCCAAATAT TTTGCAGTTGATAAACCAGCCAATCC
GmPDS1D1-233F(96)	GATCGGCTATATATCTGCTGCCAACTTCAATTATCTC GTTGGCGCCAGAAACATATCCAAATTCGCTTCTTCAG ACGCCACAATTTCGTTTTCATTTGGC
GmPDS1D1-328R(96)	GATCGCCAAATGAAAACGAAATTGTGGCGTCTGAAG AAGCGAATTTGGATATGTTTCTGGCGCCAACGAGAT AATTGAAGTTGGCAGCAGATATATAGCC

Note: (1) Underlined letters denote restriction enzymes target sites. (2) The underlines letters (GATC) represent the matching end of BamHI site. (3) Gray-highlighted letters denote the 23nt-long siRNAs predicted by MIT algorithm.

2.2 Methods

The major part of this project was carried out in the molecular plant virology laboratory, Department of Plant Pathology at Ohio Agricultural Research and Development Center (OARDC), College of Food Agricultural and Environmental Sciences, The Ohio State University.

2.2.1 Plant materials

Soybean (*Glycine max* L.) Merr cv. Williams 82 seeds used in this study were obtained from Department of Horticulture and Crop Science at OARDC, College of Food Agricultural and Environmental Sciences, The Ohio State University. Seeds were germinated in pots (three seeds per pot) and reared in greenhouse (16/8 light:dark, 25/23 °C day:night) or growth chamber (16/8 light:dark, 20 °C day:night) under light intensity of 2000 lux. The fully expanded primary leaves of eight-day-old soybean seedlings grown in greenhouse or growth chamber were inoculated with virus constructs and daily monitored for symptom development (Zhang *et al.*, 2010). Lima bean (*Phaseolus lunatus* L.) cv. Henderson-Bush seeds were also supplied by the same source mentioned above. The seeds were used in this study to prepare virus inocula (Giesler *et al.*, 2002).

2.2.2 BPMV virus strain and infectious constructs

The BPMV isolate IA-Di1 (Bradshaw *et al.*, 2007) was used in this experimental work. The BPMV RNA1 (IA-RNA1M) used in this study induces mild symptoms in soybean plants. The full cDNA sequences of BPMV RNA1 and RNA2 segments were deposited in the GenBank with accession numbers GU562879.1 and GU562880.1, respectively. The cDNAs-based RNA1 and

RNA2 constructs of BPMV were flanked by 35S promoter and terminator (P35S and T35S) of *Cauliflower mosaic virus* to yield pBPMV RNA1 and pBPMV RNA2, respectively (Zhang *et al.*, 2010).

2.2.3 Preparation of buffers and dyes

1. 50 X TAE stock solution

Tris-Base	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
ddH_2O completed to	1000 mL

The solution was stored at room temperature (25 ºC). Final (1 X) working concentration of TAE was prepared through the following equation: M1.V1=M2.V2

2. 10 X TBE stock solution

Tris-Base	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 mL
ddH_2O completed to	1000 mL

The stock solution was stored at room temperature (25 ºC).

3. 6 X DNA loading dye (50 mL)

Glycerol	15 mL
Bromophenol blue	0.125 g
Xylene cyanol FF	0.125 g
ddH_2O	35 mL

The dye solution was stored at 4 ºC.

4. Ethidium bromide (10mg/mL)

A quantity of 1 g of ethidium bromide was dissolved in 100 mL ddH_2O and stirred until the dye was completely dissolved. The dye solution was transferred to a dark bottle and stored at 4 °C. Five µL of 10 mg/mL EB was used to prepare 1.2% agarose gel so that the concentration of EB in the gel became 0.5 µg/mL.

2.2.4 Preparation of terrific broth (TB) medium

Terrific broth supplied by Fisher Scientific (USA) was used to maintain and propagate DH5α-E competent bacterial cell as follows:

Trypton	12 g
Yeast extract	24 g
K_2HPO_4	12.5 g
KH_2PO_4	2.3 g
ddH_2O	1000 mL

A quantity of 15 g/L of agar was added when needed. The medium was sterilized by autoclaving for 20 min at 121 °C and 1.05 Kg/cm^2 and poured into Petri plate and stored at 4 °C until use.

2.2.5 Preparation of ampicillin stock solution (100 mg/mL)

A quantity of 1 g of ampicillin sodium salt (Lab scientific, USA) was dissolved in 10 mL ddH_2O. The solution was filter-sterilized using 0.22 µm Millipore filter, dispensed into 2.0 mL aliquots and stored at -20 °C until use. To prepare 100 µg/mL ampicillin-containing terrific broth medium, aliquot of 1 mL of 100 mg/mL ampicillin stock was added to 1 L of the medium.

2.2.6 Preparation of super optimal broth (SOB) medium

A quantity of 20 g Trypton, 5 g Yeast extract, 0.58 g NaCl and 0.19 g KCl were added to ddH_2O to a final volume of 1 L before autoclaving at 121 °C and 1.05 Kg/cm^2 for 20 min. The pH of the medium was adjusted to 7.0 using 5 N NaOH. Just before use, then aliquot of 5 mL of sterile 2 M $MgCl_2$ was added to the already sterilized SOB medium.

2.2.7 Bacterial transformation (Sambrook and Russell, 2001)

DH5α competent *E. coli* cells (Invitrogen, USA) were transferred from the deep freezer (-80) °C and thawed on ice. After thawing, the cell suspension was gently mixed with the pipette tip. For each individual transformation, 100 µL aliquots cell suspension were transferred into 1.5 mL tubes that were pre-chilled on ice. Aliquot of 2 µL (10 ng) plasmid DNA or 10 µL (50 ng) of DNA ligation product was added to the cells and mixed gently. The tubes were incubated on ice for 30 min. The cells were heat-shocked at 42 °C for exactly 45 sec without shaking, and then the tubes were then kept on ice for 2 min. Aliquot of 400 µl of pre-warmed (37 °C) SOB (without antibiotics) was added to each tube before shaking at 37 °C for 1 h. After shaking, 50 µL aliquot plasmid- or 200 µL of ligation product-transformed cells were spread onto ampicillin-containing TB plates. The plates were kept at 37 °C overnight.

2.2.8 Plasmid propagation, purification, nucleotide sequencing, restriction enzyme digestion, dephosphorylation and ligation

All plasmids were propagated in DH5α cells and purified using the ZR Plasmid Miniprep™-Classic kit (Zymo Research, USA). Nucleotide sequencing was done in the Molecular and Cellular Imaging Center (MCIC), OARDC, The Ohio State University using The Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, USA) and the ABI Prism 3100xl genetic

analyzer. All the restriction enzyme digestion, dephosphrylation and ligation of cloning vectors in this experimental work were performed using restriction enzymes, T4 ligase and FastAP thermosensitive alkaline phosphatase, respectively, manufactured by Thermo scientific (Fermentas, USA) according to the manufacturer's protocol.

2.2.9 DNA extraction from gel and cleaning of cloning vectors and inserts

The cloning vectors were linearized with restriction enzyme(s) and gel purified to remove undesired inserts, to be prepared for ligation. The linearized cloning vectors did not contain any insert and/or PCR products needed to be digested or ligated were cleaned before setting ligation. The gel DNA recovery or cleaning of DNA samples were performed using Zymoclean™ gel DNA recovery kit or DNA clean and concentrator™-5 kit (Zymoresearch, USA) according to the manufacturer's instructions.

2.3 BPMV RNA2-derived VIGS constructs with nonviral inserts

The pBPMV RNA2-based VIGS vectors (V1 and VM) used in this study were developed by Zhang *et al.* (2010). The V1 is characterized by having BamHI restriction enzyme site within the 3' untranslated region (UTR) of its RNA2 for insertion of nonviral inserts, whereas the VM vector was developed to carry inserts between the movement protein (MP) and large coat protein (L-CP) of the RNA2.

2.3.1 Designing the 27 nt-long sequences derived from GmPDS1 for inserting into BPMV RNA2 V1 vector

The full length cDNA of *G. max* Phytoene desaturase1gene (GmPDS1) was obtained from the GenBank (accession no. NM_001249840.1). Three best siRNAs (GmPDS1a, b and c) of 23nt-long were predicted by submitting the cDNA sequence of GmPDS1 to the MIT algorithm (sirna.wi.mit.edu) to find

the best siRNA targeting site. The lengths of siRNA sequences were extended two nucleotides beyond each of 5' and 3' of the 23nt-long predicted siRNAs to make the actual length of each siRNA 27nts. The resulting fragments in sense direction that designated as GmPDS1a-2052F, GmPDS1b-1353F and GmPDS1c-1529F corresponded to nt #2052-2078, 1353-1379 and 1529-1555 of GmPDS1, respectively. The antisense siRNAs designated as GmPDS1a-2078R, GmPDS1b-1379R, and GmPDS1c-1555R were complementary to GmPDS1a-2052F, GmPDS1b-1353F and GmPDS1c-1529F, respectively, as shown in table 1. To insert these siRNA sequences into the V1 vector using *Bam*HI (GGATCC) cloning site, four nucleotides (GATC) of *Bam*HI site were added at the 5' end of both sense and antisense complement strands of the siRNAs. The siRNA inserts were supplied by Sigma-Aldrich, USA.

2.3.2 Designing the 96nt-long sequences derived from GmPDS1 inserting into BPMV RNA2 V1 vector

A 325-nt insert derived from GmPDS1cDNA and corresponding to nt #1518-1843 was already inserted in an antisense orientation into the BPMV RNA2-based V1 vector that developed by Zhang *et al.* (2010). The vector used in this study was designated as a positive control for the silencing of GmPDS1 (pos. ctrl. V1-GmPDS1).

The sequence of the 325nt-long fragment of GmPDS1 was used as a template to find the best siRNA targeting site using MIT algorithm. The resulting 23nt-long siRNA corresponding to nt #1531-1551 of GmPDS1cDNA (the highlighted letters within GmPDS1A1-1494F) as shown in table 1. To design the first 96nt-long fragment derived from GmPDS1, the predicted siRNA was placed in the middle of 96nts of GmPDS1. The yielded sense and antisense 96nt-long fragments corresponding to nt #1494F-1589R were designated as GmPDS1A1-1494F and GmPDS1A1-1589R, respectively (Table 1).

The second 96-nt fragment was selected to target the region spanning from nt #1683F-1778R that is outside the best siRNA-targeting site (as predicted by MIT algorithm). The sense and antisense complementary fragments were designated as GmPDS1B1-1683F and GmPDS1B1-1778R, respectively (Table 1).

The third 96 nts fragment was designed to have targeting site within the entire coding region (CDS) of GmPDS1. CDS of GmPDS1 was submitted to MIT algorithm for predicting the best siRNA-targeting site within the CDS of GmPDS1. The predicted siRNA sequence of 23nt-long corresponded to nt #576-597 of GmPDS1 cDNA (the highlighted letters of GmPDS1C1-539F, table 1). As designated as GmPDS1C1-539F, the corresponding 96-nt fragment flanking the predicted siRNA target site spanned from nt #539-634 of GmPDS1. The antisense complementary fragment was designated as GmPDS1C1-634R, as shown in table 1.

The second best siRNA-targeting site predicted also by MIT algorithm and corresponded to nt #270-292 was used to design the fourth 96-nt fragment, GmPDS1D1-233F, targeting against GmPDS1 CDS (Table 1). The resulting fragment covered the region spanning from nt #233-328 of GmPDS1 cDNA. The antisense complementary was designated as GmPDS1D1-292R, as shown in table 1. The four nucleotide matching end, GATC, of *Bam*HI restriction site was included into the 5' end of each sense and antisense 96-nt long fragment for insertion into the BPMV RNA2-based V1 vector.

2.3.3 Construction of V1-based VIGS vectors and screening the constructs

Self-annealing (sense and complementary antisense) fragment pairs, GmPDS1a F-R, GmPDS1b F-R, GmPDS1c F-R, GmPDS1A1 F-R, GmPDS1B1 F-R, GmPDS1C1 F-R, or GmPDS1D1 F-R, corresponding to distinct regions of GmPDS1 cDNA were annealed for cloning (Table 1). The

annealing reaction was as follows: 1 µL of each 20 µM sense and antisense complement oligo, 2 µL of 10X T4 DNA ligase buffer and 16 µL of nuclease-free water to bring the total volume to 20 µL. All tubes were incubated in thermocycler under the following conditions: 95 ºC, 5 min; 65 ºC, 5 min; 35 ºC, 5 min; 25 ºC, 5 min. Annealing was done at the end of 25 ºC incubation. The concentration of annealed oligo pair became 1 µM.

Aliquot of 1 µL of each annealed oligo that has the *Bam*HI matching end (GATC) at both 5' ends was ligated into 50 ng of *Bam*HI digested and dephosphorylated BPMV RNA2-based V1 vector. The resulting constructs were designated as V1-GmPDS1a, V1-GmPDS1b, V1-GmPDS1c, V1-GmPDS1A1, V1-GmPDS1B1, V1-GmPDS1C1, and V1-GmPDS1D1 and their GmPDS1 inserts were inserted in antisense (reverse) orientation.

To screen the clones mentioned above, as an initial step for the success of the ligation, the BamHI site was lost after ligation, thus making them to be undigested by BamHI. Insertion orientation of V1-GmPDS1a, V1-GmPDS1b, or V1-GmPDS1c was confirmed by PCR using primer pairs BPMVII-2937F as a forward primer and GmPDS1aF, bF, or cF, respectively, as a reverse primer to characterize their counterpart inserts. The orientation of V1-GmPDS1A1, V1-GmPDS1B1, V1-GmPDS1C1, or V1-GmPDS1D1 was confirmed by PCR using BPMVII-2937F as a forward primer and GmPDS1-1499F, GmPDS1-1684F, GmPDS1-540F or GmPDS1-234F, respectively, as a reverse primer (Table 1). Sequencing of the constructs was done by using the primer BPMVII-2937F.

2.4 Developing R2G-460BamHI mutant

A set of overlapping PCRs was performed to introduce four nts (ATCC) after nt #461 of the cDNA of RNA2 5' UTR, creating *Bam*HI site. As an RNA2 surrogate, cDNA of RNA2 containing a GFP insert between MP and L-CP

(RNA2 GFP or R2G) was used to facilitate the tracking of the infection process (Zhang *et al.*, 2010). PCR A was done by using R2G cDNA as a template and primer pair BR2G-460*Bam*HI-F and BRIIG-904R (*Sal*I) listed in table (1). PCR B was performed with R2G cDNA as a template and primer pair BPMVII-1F (*Xho*I) and BR2G-460*Bam*HI-R listed in table (1). PCR C was performed using PCR A and B products as template and primer pair BPMVII-1F (*Xho*I) and BRIIG-904R (*Sal*I). The PCR C product was digested with *Xho*I and *Sac*I, gel extracted and ligated into similarly digested and gel extracted R2G vector. The resulting mutant was referred to as R2G-460*Bam*HI. This mutant along with pBPMV RNA1 was co-bombarded into lima bean cotyledons and inspected under microscope three days after bombardment to detect the mutant replication through GFP fluorescence. Soybean plants were subsequently rub-inoculated using the bombarded cotyledons to further assess the mutant infectivity.

2.5 Modification of 5' UTR of BPMV RNA2

A set of overlapping PCRs was performed to delete the region spanning from nt #263 to #309 within the 5' UTR of BPMV RNA2 and replace it with the *Bam*HI, *Acc*65I and *Eco*72I restriction sites. PCR A was performed with pBPMV RNA2 as template and primer pair BPMVII-1F (*Xho*I) and BPMV-V5UE-R (Table 1). PCR B was performed using pBPMV RNA2 as template and primer pair BPMV-V5UE-F and BRIIG-904R (*Sal*I) listed in table 1. PCR C was performed with PCR products of A and B as template and primer pair BPMVII-1F (*Xho*I) and BRIIG-904R (*Sal*I). The product of PCR C was digested with *Xho*I and *Sac*I. The digested PCR C product was gel extracted and ligated into similarly digested pBPMV RNA2 to yield V5UE. The introduction of the restriction sites (*Bam*HI, *Acc6*5I and *Eco*72I) was initially confirmed by digestion with either *Xho*I and *Bam*HI or *Acc*65I and *Sac*I. The

sequence identity of the insert was confirmed by nucleotide sequencing using BPMVII-1F (*Xho*I) primer (Table 1). The PCRs were performed as follows:

1. Aliquot of 1 µL (5 ng/µL) of pBPMV RNA2 was used as template.
2. Equal volumes of forward and reverse primers of 20 µM were mixed, so that the final concentration of each primer in the mix was 10 µM. Aliquot of 1 µL of the mix was used in a 20 µL PCR reaction.
3. 10 µL of 2x PCR master mix (Phusion® Hot Start II High-Fidelity DNA Polymerase, Finnzymes, USA) that contains DNA polymerase, dNTPs, and necessary salt conditions.
4. The total volume of PCR reaction was brought to 20 µL by nuclease-free water.
5. The PCR conditions were as follows: one cycle of denaturing at 98 ºC for 30 s, 30 cycles of denaturing at 98 ºC for 10 sec, annealing at 65 ºC for 10 sec, extending at 72 ºC for 45 sec; and an extra 10 min of extending at 72 ºC.

2.5.1 Construction of V5UE-based VIGS constructs

Three different-sized fragments of soybean origin were selected to be inserted into BPMV RNA2 V5UE mutant. The first insert, GmPDS1, was 325 nts corresponding to nt #1518-1843 of GmPDS1 cDNA. Since this fragment was shown by Zhang *et al.* (2010) to induce extensive photobleaching in soybean leaves, due to gene silencing of GmPDS1, when inserted within the 3' UTR (V1-GmPDS1) or between MP and L-CP (VM-GmPDS1) of BPMV RNA2, it was chosen to provide visual indicator of virus viability. The *Bam*HI and *Acc*65I sites were introduced into the 5' and 3' ends of GmPDS1 insert respectively. All the AUG start codons were removed to avoid extra translational initiation. The 325nt-long GmPDS1 fragment was digested with *Bam*HI and *Acc*65I and ligated in the antisense direction into the similarly digested V5UE vector to yield V5UE-GmPDS1.

The second and third inserts, GmDCL2 and GmDCL4 were 345 and 300nt-long respectively. The cDNA sequences of GmDCL2 and GmDCL4 were obtained from nt collection of GenBank (ncbi.nim.nih.gov). The *Arabidopsis thaliana* DCL2 and DCL4 cDNA amino acid sequence were used as query to search for soybean nt collections. Four soybean cDNAs were identified. Two sequences were potential soybean DCL2 orthologs. They are GmDCL2a (XM_003534726.1) and GmDCL2b (XM_003535056.1). The other two sequences were potential soybean DCL4 orthologs, GmDCL4a (XM_003541423.1) and GmDCL4b (XM_003550749.1). The GmDCL2 or GmDCL4 orthologs were highly homologous but not identical. They encoded uninterrupted proteins.

The MIT algorithm was used to predict two best siRNAs, GmDCL2-siRNA and GmDCL4-siRNA, which could target the soybean cDNA orthologs, GmDCL2 and GmDCL4, respectively (Table 1). The selected GmDCL2 fragment corresponds to nt #1811-2154 of the full length GmDCL2 cDNA, while that of GmDCL4 corresponds nt #2720-3019 of full length GmDCL4 cDNA. The GmDCL2 insert (345nts) was 45nts longer than GmDCL4 (300nts) to facilitate characterization. GmDCL2 and GmDCL4 inserts were designed flanked with *Acc*65I site (GGTACC) at 5' and 3' ends. Both inserts were modified to remove the undesired AUG start codons. The GmDCL2 or GmDCL4 fragment was inserted, in the antisense orientation, downstream the GmPDS1 insert of *Acc*65I-linearized and phosphorylated V5UE-GmPDS1 mutant. The total sizes of the resulting inserts GmPDS1/GmDCL2 and GmPDS1/GmDCL4 were 670 and 625nts, respectively. Sequencing of the constructs was done with the primer BPMVII-1F (*Xho*I) listed in table (1). The yielded mutants, V5UE-GmPDS1/GmDCL2 and V5UE-GmPDS1/GmDCL4, were used to silence the GmPDS1/GmDCL2 and GmPDS1/GmDCL4, respectively.

2.6 Preparation of plant tissue culture medium

The components of OMS culture medium containing MS salts (Murashige and Skooge, 1962), B5 vitamins (Gamborg *et al.*, 1968), 3% sucrose and 0.2% Gelrite were used (Table 2). MS salts were grouped into four categories, sulfates, halides, FeEDTA, and P, B, Mo. Stock solutions (100x) were prepared from each group. B5 vitamins were also prepared into 100x stock solution for the preparation of 1x OMS culture medium (Table 3). No (PGRs) were added. The 1x OMS medium was sterilized by autoclaving at 121 ^{0}C and 1.05 Kg/cm^2 for 20 min.

Table 2. Composition of the OMS medium used in the experimental work (Garcia-Hernandez *et al.*, 2010).

Major elements		**Minor elements**	
Salts	**mg/L**	**Salts**	**mg/L**
NH_4NO_3	1650	H_3BO_3	6.2
KNO_3	1900	$MnSO_4.4H_2O$	22.3
$CaCl_2.2H_2O$	440	$ZnSO_4.4H_2O$	8.6
$MgSO_4.7H_2O$	370	KI	0.83
KH_2PO_4	170	$Na_2MoO_4.2H_2O$	0.25
Na_2-EDTA	37.3	$CuSO_4.5H_2O$	0.025
$FeSO_4.7H_2O$	27.8	$CoCl_2.6H_2O$	0.025
Organic constituents			
Components	**mg/L**	**Components**	**Amount/L**
Nicotinic acid	1	*myo*-Inositol	100 mg
Thiamine. HCL	10	Sucrose	30 g
Pyridoxine. HCL	1	Gelrite	2 g

Table 3. Preparation of 1x OMS medium from 100x stock solutions of MS salts and B5 vitamins.

Stock solutions and other ingredients	1x OMS (1000) mL	Stock solutions and other ingredients	1x OMS (1000) mL
MS FeEDTA	10 mL	NH_4NO_3	1.65 g
MS sulfates	10 mL	KNO_3	1.9 g
MS halides	10 mL	Sucrose	30 g
MS P, B, Mo	10 mL	Gelrite	2 g
B5 vitamins	10 mL		

2.7 Preparation of virus inoculation buffer (Lin *et al.*, 2013)

Phosphate buffer and celite 545-AW were used for preparation of the virus inoculation buffer. A phosphate buffer (0.05 M), pH 7 was made by adding 0.6 g of sodium phosphate dibasic (Na_2HPO_4) and 6.4 g of sodium phosphate monobasic (NaH_2PO_4) to a total volume of 1 liter ddH_2O. Aliquot of 100 mL of the already prepared phosphate buffer was dispensed into a 200 mL-glass reagent bottle, and then a quantity of 1 g of celite 545-AW was added. The inoculation buffer was sterilized by autoclaving at 121 °C and 1.05 Kg/cm^2 for 20 min and stored at -20 °C until use.

2.7.1 Preparation of virus inocula

The following methodology outlines a protocol for preparation of virus inocula for inoculating soybean plants. The procedure was slightly modified and divided into three steps as follows:

I. Seed preparation (Garcia-Hernandez *et al.*, 2010)

Magenta™GA7 containers were prepared for germinating lima bean (*Phaseolus lunatus* cv. Henderson-Bush) seeds. Filter papers or paper towels were folded to fit in the bottom of Magenta boxes. About 25 mL of ddH_2O

were added to each container to moisten paper towels. The boxes containing moistened paper towels were autoclaved for 20 min. Using 50 mL disposable centrifuge tubes, lima bean seeds were disinfected with a 4% (v/v) commercial bleach solution (30 seeds in 20-40 mL) for 20 min with shaking on a gyratory shaker at 60 rpm. After shaking, the seeds were moved to a laminar flow hood for further processing. The seeds were rinsed 3-5 times with sterile ddH_2O with gentle agitation for 30 sec during each rinse. Sterile seeds (7-8) were placed between layers of folded paper located in each sterile magenta box, then for 4 days at 25 ^{0}C and 16/8 h light:dark.

II. Introduction of virus constructs using particle bombardment (Garcia-Hernandez *et al.*, 2010)

The pBPMV RNA2-based mutants were already prepared for introducing them into lima bean cotyledons using particle bombardment. Approximately 1-2 hrs before bombardment, lima bean cotyledons were brought to laminar air flow hood and excised from germinating seedlings and the seed coats were removed. Excised cotyledons were placed on OMS culture medium. The pBPMV RNA2 cDNA-based construct that mixed with pBPMV RNA1 cDNA in equal ratios were precipitated onto M10 tungsten particles (Sylvania, USA). Using 0.6 mL microfuge tube, 25 µL tungsten particles (particles were suspended in sterile water, 100 mg/mL, right before use), 10 µL pBPMV RNA1 (400 ng/µL), 10 µL pBPMV RNA2-based construct (400 ng/µL), 25 µL of 2.5 M calcium chloride and 10 µL of 100 mM spermidine were mixed (modified). The components were mixed thoroughly by brief vortexing. The DNA preparation was incubated on ice for 5 min, and then 50 µL supernatant was removed and discarded. The DNA-coated particles were suspended by vortexing and 2 µL aliquot was immediately removed (this vortexing step should be repeated every time a 2 µL aliquot is removed from the tube). Coated particles were kept in ice and used within 1 min. Through the top of a syringe

filter, aliquot of 2 µL of coated particles were placed in the middle of the filter screen. The filter unit containing the coated particles was placed in the filter holding unit inside the particle gun chamber. A lima bean cotyledon was placed, adaxial side up, on a baffle that then placed in the particle gun chamber. The baffle, which consists of a screen melted to the bottom of a beaker, was used as a platform support tissues during bombardment. The bombarded cotyledon, adaxial side up, was returned to OMS culture medium. A minimum of three cotyledons were bombarded for each construct. The BPMV mutants-bombarded cotyledons were incubated at 25 ^{0}C and 16/8 light:dark for 3 days for virus propagation.

III. Inoculation of soybean plants with virus inocula

Soybean seeds (3-5 per pot) were sown in 12 cm (5-inch) pots and placed in growth chamber or greenhouse. The deformed or damaged seedlings were discarded so that each pot contained three healthy plants. Three pots (each pot contained three soybean plants) were labeled for each virus construct inoculation (treatment). The emerging soybean seedlings that were ready to inoculate should contain expanded primary leaves and the subsequent leaves should not be obvious. The primary leaves of eight day-old soybean seedlings were labeled with marker pen, to be ready for virus inoculation (Lin *et al.*, 2013). BPMV-based mutant-bombarded cotyledon (one cotyledon was enough to inoculate 15 soybean plants) was crushed and homogenized with 1 mL inoculation buffer using sterile mortar and pestle (the unused bombarded cotyledons were stored at -80 ^{0}C for further use). Aliquot of 25 µL aliquot of the virus inoculum was applied on the leaf surface using micropipette. The leaves were lightly rubbed, distributing the inoculum over the entire surface. The inoculated plants were reared in growth chamber or greenhouse and monitored daily for the appearance of virus symptoms. Virus-infected plant materials were discarded by autoclave after finishing each experiment.

2.8 Total RNA extraction from plant tissue

About 0.3 g of plant tissue (soybean leaf) was thoroughly ground to powder in liquid nitrogen. Aliquot of 1 mL of 4 ºC Tripure™ reagent (Roche, USA) was added to the powder with continuous mixing until thawing of paste. The thawed material was poured into a 2 mL Eppendorf tube and left at room temperature (25 ºC) while working on the rest of samples. After adding 600 µL of chloroform, the contents were mixed twice by vortex for 20 and 10 sec each. The tubes were centrifuged at 12.000 xG, 4 ºC, for 15 min. The aqueous phase (700-800 µL) was transferred to 1.5 mL tube, without touching the middle phase. Aliquot of 600 µL isopropanol was added to the tubes containing the supernatant and the contents were mixed by inverting the tubes for 50 times. After centrifugation at 12.000 xG, 4 ºC, for 15 min, the supernatant was drained. The pellet was washed with 750 µL 75% ethanol, vortexing to loosen the pellet, and spinning at 12.000 xG, 4 ºC for 5 min to re-sediment the pellet. The ethanol was drained and the pellet was left for 10 min to air-dry. The dried pellet was dissolved in 50 µL of nuclease-free water. After determination of RNA concentration using NanoDrop, a quantity of 5 µg of RNA was run on a 0.5 X TBE gel to ensure the integrity of the RNA.

2.8.1 Reverse transcriptase (RT-PCR) protocol

The RNA samples that isolated in the above protocol were subjected to reverse transcriptase RT-PCR. The following protocol was divided into three steps as follows:

I. DNase treatment

The RNA samples were treated with DNase I using DNA-free reagents (Ambion, USA) as follows:

RNA (10 µg, volume adjusted with 40 µL of RNase-free water)	40 µL
10 X DNase I buffer	5 µL
rDNase I (2 U/µL)	1 µL
RNase-free water	4 µL
Total	50 µL

The mixture was incubated at 37 ^{0}C for 30 min, and then aliquot of 5 µL of DNase inactivation reagent (provided by Ambion as a component of the DNA-free kit) was added, mixed well, and incubated at room temperature for 2 min. The mixture was centrifuged for 2 min at 12.000 xG, and then 45 µL of RNA solution was carefully removed into fresh tube (s). Aliquot of 5 µL (1 µg) of each RNA sample was run on gel to ensure the integrity of the DNase-treated RNA.

II. cDNA synthesis

This step included the synthesis of cDNAs from RNA as a template and comprised of two steps, annealing and reverse transcription.

a. Annealing

For each RNA sample, aliquot of 20 µL annealing mixture in a 0.5 mL was prepared using the following components:

DNase-treated RNA	5 µL (1 µg)
Oligo-dT primer (100 ng/µL, see table 4)	2 µL
10 mM dNTP mix	2 µL
RNase-free water	11 µL
Total	20 µL

The annealing mixture was incubated at 65 ^{0}C for 5 min, and cooled on ice.

b. Reverse transcription

Two cDNA synthesis mixtures were prepared, with RT (RT+) and without (RT-).

Components	RT+	RT-
5 X RT buffer	4 µL	4 µL
100 mM DTT	2 µL	2 µL
RT (200 U/µL, Clontech, USA)	0.5 µL	—
Annealing mixture	10 µL	10 µL
RNase-free water	3.5 µL	4 µL
Total	20 µL	20 µL

The cDNA synthesis was performed using thermocycler at the following conditions: 42 ^{0}C for 60 min followed by 70 ^{0}C for 15 min. The resulting RT products were diluted to 1:5 by adding 80 µL RNase-free water.

III. PCR

Forward and reverse primers for each of the cDNAs to be amplified were mixed to minimize the pipetting at the stage of PCR set up. For PCR purpose, the concentration of each primer in the mix should be 10 µM. One µL of the mix was used in a 20 µL reaction so that the final concentration of each primer was 500 nM. The primers listed in table (4) were used for RT-PCR amplification of GmACT1, GmPDS1, GmDCL2, GmDCL4, BPMV RNA1, or BPMV RNA2 mRNA transcripts.

Table 4. Primers used for RT-PCR amplification

Primer Name	Primer Sequence (5'→3')
Oligo $(dT)_{25}$	TTTTTTTTTTTTTTTTTTTTTTTTT
GmACT1-136F	GTAGTTGGTATGGGCCAGAAAGA
GmACT1-481R	CACCATCCCCAGAATCCAACACA
GmPDS1-938F	CCATATGTTGAGGCTCAAGATG
GmPDS1-1464R	AGGTGATCATATGTGTTCTTCAG
BPMV-I/II-68F	GGAAACAAAAGCAATCGTTAC

BPMVII-MP-KO-R	GTTTCTAATCACTTCAAGCTTTTCCTCTTC
BPMVR1-410F	ACTTTCAGAGTAATGAGACAGCCAA
BPMVR1-1204R	TGATCAATTCACTGTTCGTGACGAA
GmDCL2a-3619F	CCTGAAGTTCCTAGATGTTATCAG
GmDCL2a- 4072R	CATTCAACTCTCGGATGGGATG
GmDCL4a-3180F	CCTTCATGAAGGAGACCTTACAA
GmDCL4a-3808R	GCTTTAGTTTGGGATAAGCTGAGA

A 2 X PCR master mix, Lucigen Econo Taq Plus Green, USA, that contains Taq polymerase, dNTPs, and necessary salts was used. Two PCR reactions were performed for each sample having RT+ and RT- reactions as follows:

Template (RT product)	5 µL
Primer mix	1 µL
2 X PCR master mix	10 µL
RNase-free water	4 µL
Total	20 µL

The PCR conditions were adjusted as follows: one cycle of denaturing at 94 ºC for 2 min, 27 cycles of denaturing at 98 ºC for 15 sec, annealing at 58 ºC for 15 sec, extending at 72 ºC for 30 sec; and an extra 10 min of extending at 72 °C.

Chapter Three

Results and Discussion

3. Results and discussion

3.1 GmPDS1-derived inserts of 96 nts can initiate sporadic photobleaching in systemic soybean leaves

To determine whether the PTGS of an endogenous gene could be initiated by homologous short regions in BPMV-based VIGS vector, oligonucleotides were designed to the different regions of the endogenous soybean (*Glycine max*; Gm) phytoene desaturase 1 (GmPDS1). The cDNAs of BPMV RNA1 and RNA2 were flanked by the 35S promoter and terminator (P35S and T35S) of *Cauliflower mosaic virus* as shown in fig. 3.1A. The BPMV RNA1 (IA-RNA1M) that induces mild symptoms was used to support the replication of the RNA2-based mutants and to prepare the viral inocula. The BPMV RNA2-based vector, V1, was used to carry the inserts of the non-viral origin. As a positive control (pos.ctrl. V1-GmPDS1), 325 bp cDNA fragment corresponding to the 3'UTR of GmPDS1 was cloned in an antisense orientation into *Bam*HI restriction site within the 3'UTR of the V1 vector and used (Zhang *et al.*, 2010; Fig. 3.1B and C). Three short cDNA fragments with similar sizes (27nt) of GmPDS1 were predicted by the Massachusetts Institute of Technology (MIT) algorithm and inserted into the V1 vector. These three GmPDS1-derived fragments, GmPDS1a, b, or c, correspond to three distinct regions of 3' open reading frame (ORF) of GmPDS1 (Fig. 3.1C). GmPDS1a, b, or c were inserted within the 3'UTR of the V1 mutant in an antisense direction using *Bam*HI restriction site to produce V1-GmPDS1a, b, and c constructs respectively as shown in fig. 3.1B.

Equal ratios of RNA1 and RNA2-based cDNA constructs were used to inoculate lima bean cotyledons with particle bombardment to prepare the viral inocula. Three days after bombardment, the treated cotyledons were

homogenized and used to inoculate the primary leaves of eight-day-old soybean plants.

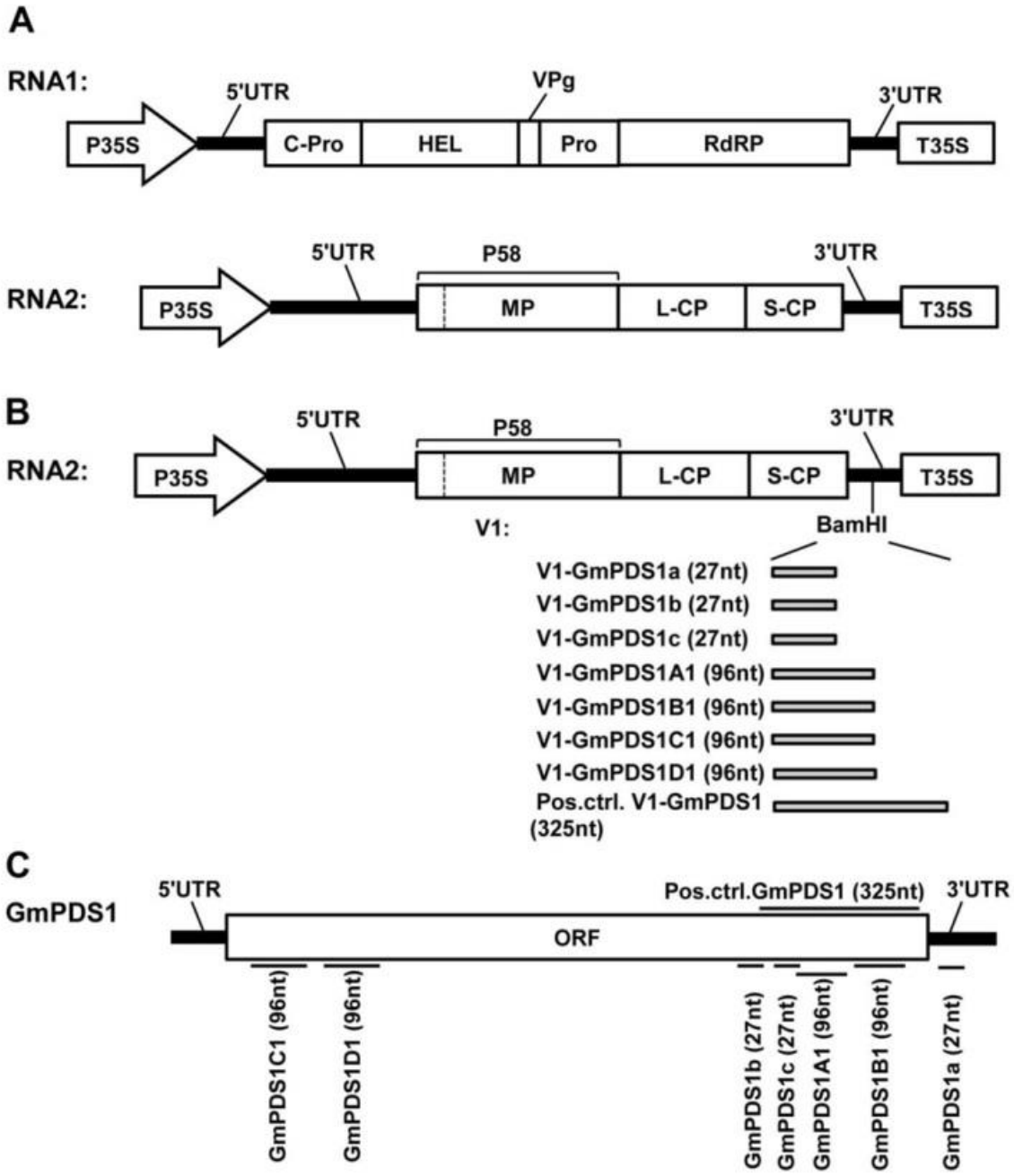

Figure 3.1: Schematic representations of BPMV RNA1 and RNA2 constructs used in this study. A: the top diagram depicts the full length cDNA of RNA1 (BPMV-IA-RNA1M) flanked by CaMV 35S promoter and terminator (P35S and T35S), used to support RNA2 replication. The long open box denotes the single open reading frame (ORF) encoded by RNA1, with vertical lines depicting the boundaries of five mature proteins released by protease (Pro)-mediated processing. C-Pro: putative protease cofactor; HEL: putative helicase; VPg: viral protein-genome-linked; RdRP: RNA-dependent RNA polymerase. Similarly, the RNA2-encoded polyprotein is processed into P58, movement protein (MP), large and small coat protein subunits (L-CP and S-CP). B: the BPMV RNA2-based V1 vector has *Bam*HI site at its 3' UTR, used to insert cDNA of seven

GmPDS1-derived fragments of distinct lengths. The positive control (Pos.ctrl.) V1-GmPDS1 with a 325 nt-long of the GmPDS1 reported in an earlier study (Zhang *et al.*, 2010). C: the schematic representation of the full length cDNA of soybean phytoene desaturase (GmPDS1). The bold lines with various lengths depict the fragments corresponded to different regions of the GmPDS1 and inserted into the V1 vector.

The inoculated soybean plants were grown in a growth chamber or greenhouse and monitored daily for symptom development. Mock-inoculated control denotes healthy plants that inoculated only with an inoculation buffer (Fig. 3.2A and G). The viral symptoms on systemically infected leaves started as early as eight days post inoculation (8 dpi) as shown in fig. 3.2B and H. Compared with the leaves of plants infected with the pos.ctrl. V1GmPDS1 (Fig. 3.2C and I), the leaves of plants infected with the V1-GmPDS1a, b or c mutant and grown in the growth chamber or in the greenhouse failed to show photobleaching, indicating that the expression of GmPDS1 would not be down regulated (Fig. 3.2D, E, F, J, K, and L, respectively). The growth-chamber and greenhouse-grown plants infected with the pos.ctrl. V1-GmPDS1 showed photobleaching on systemically infected leaves, indicating successful silencing of GmPDS1 expression (Fig. 3.2C and I, respectively). Interestingly, the leaves of plants grown in the growth chamber and infected with all V1-derived constructs, V1-empty vector, pos.ctrl. V1-GmPDS1 or V1-GmPDS1a, b or c, were severely symptomatic with moderately blistered and deformed leaves (Fig. 3.2B, C, D, E and F, respectively). By contrast, the infection of plants reared in the greenhouse with the same category of the virus mutants showed mild symptoms with slightly deformed leaves (Fig. 3.2H, I, J, K and L), respectively. Thus, the growth conditions might affect the symptom severity of the virus infection.

To summarize these findings, all of 27nt-long GmPDS1 derivatives, GmPDS1a, b, and c, were unable to direct GmPDS1 silencing in soybean plants grown in growth chamber or greenhouse, although they targeted distinct regions of the ORF of GmPDS1. Their sizes might not be sufficient to induce

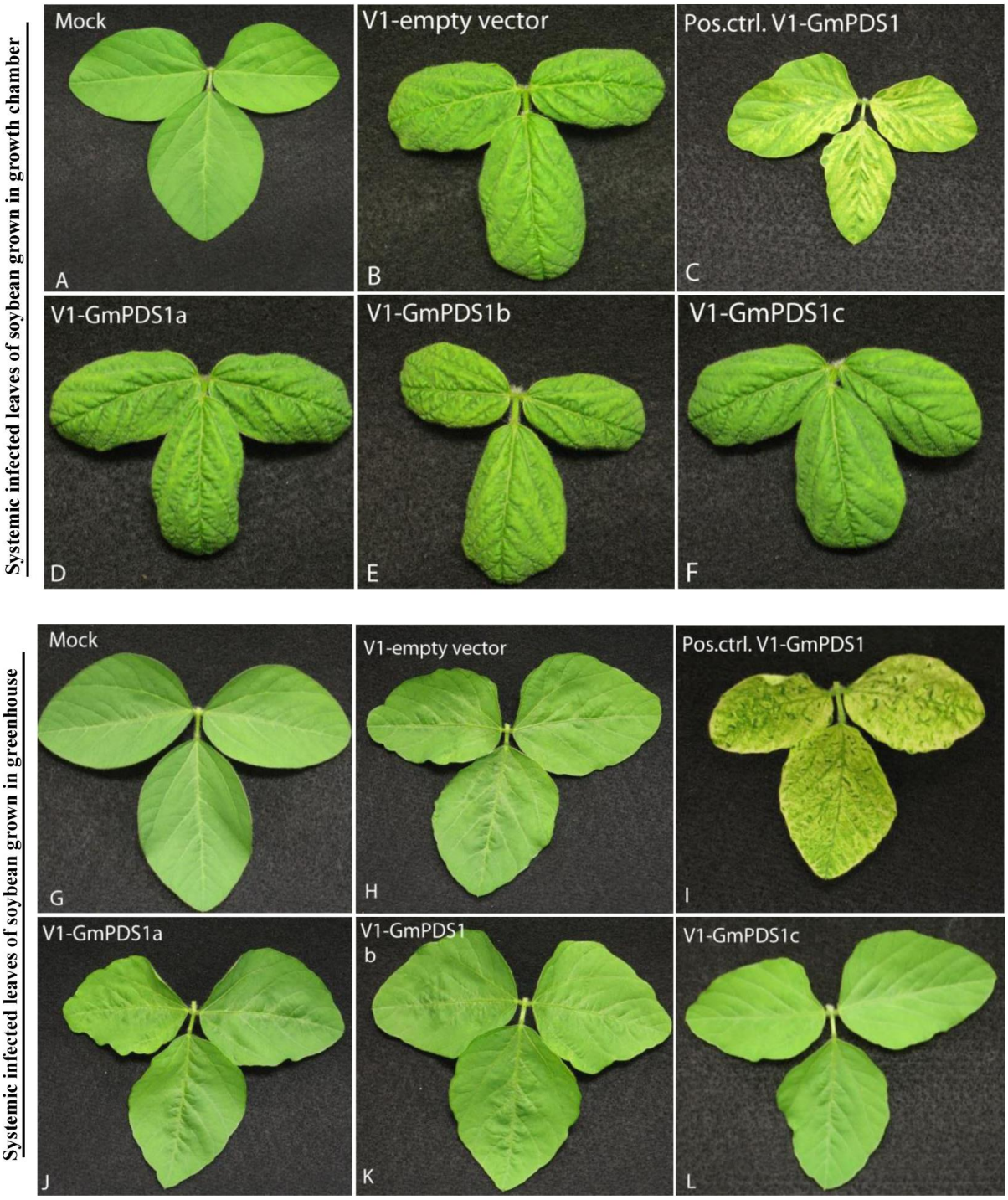

Figure 3.2: The V1 mutants contain 27 nts of soybean-derived inserts tested at different growth conditions. A and G: leaves of healthy control soybean plants inoculated with only inoculation buffer. B-F: systemic leaves of soybean plants grown in growth chamber and infected with the five mutants. H-L: systemic infected leaves of plants grown in greenhouse. C and I: soybean leaves infected systemically with a V1 mutant containing 325 nts of GmPDS1. BPMV-IA-RNA1M was used as the RNA1 clone for all inoculations Images were taken at 15 dpi.

virus-based GmPDS1 silencing. The greenhouse conditions might have been more convenient for virus multiplication and GmPDS1 silencing in plants infected with the pos.ctrl. V1-GmPDS1.

Silencing of PDS results in suppression of carotenoid biosynthesis so that the affected plants become susceptible to photobleaching. The GmPDS1 was selected to easily provide visual indicator of PDS silencing. The ability of homologous inserts longer than 30nt to induce silencing of PDS was reported in *Nicotiana benthamiana* (Thomas *et al.*, 2001). Cloning of fragment in an antisense orientation effectively induces silencing efficiency regardless of the fragment size (Thomas *et al.*, 2001; Zhang and Ghabrial, 2006; Zhang *et al.*, 2010). Therefore, the GmPDS1a, b, or c was inserted in antisense direction.

Plant growth conditions could alter its susceptibility to the virus infection (Qu *et al.*, 2005). The infectivity of BPMV is attributed to its RNA1 (Gu and Ghabrial, 2005). In spite of using the RNA1 (IA-RNA1M) that induces mild symptoms, plants reared in the growth chamber showed increased severity to the virus infection. The disadvantage of severe virus isolates to be used as VIGS vectors is that their severe symptoms are more likely to interfere with phenotype caused by the gene silencing than that of the mild ones (Zhang *et al.*, 2009). This has led to speculate the moderately severe symptoms of plants grown in the growth chamber might influence the efficiency of GmPDS1 silencing caused by the pos.ctrl. V1-GmPDS1 compared with the same set grown in the greenhouse.

To test the feasibility of VIGS in promoting GmPDS1 silencing using V1 vectors that contain inserts shorter than 100 nt, fragments of 96nt-long that designed to be targeted against distinct regions of GmPDS1 were designated GmPDS1A1, B1, C1 and D1 (Fig. 3.1C). These fragments were selected to

determine the minimum size of insert required to initiate silencing of the GmPDS1. However, the photobleaching in plants treated with these inserts should be similar to pos.ctrl V1-GmPDS1-infected plants.

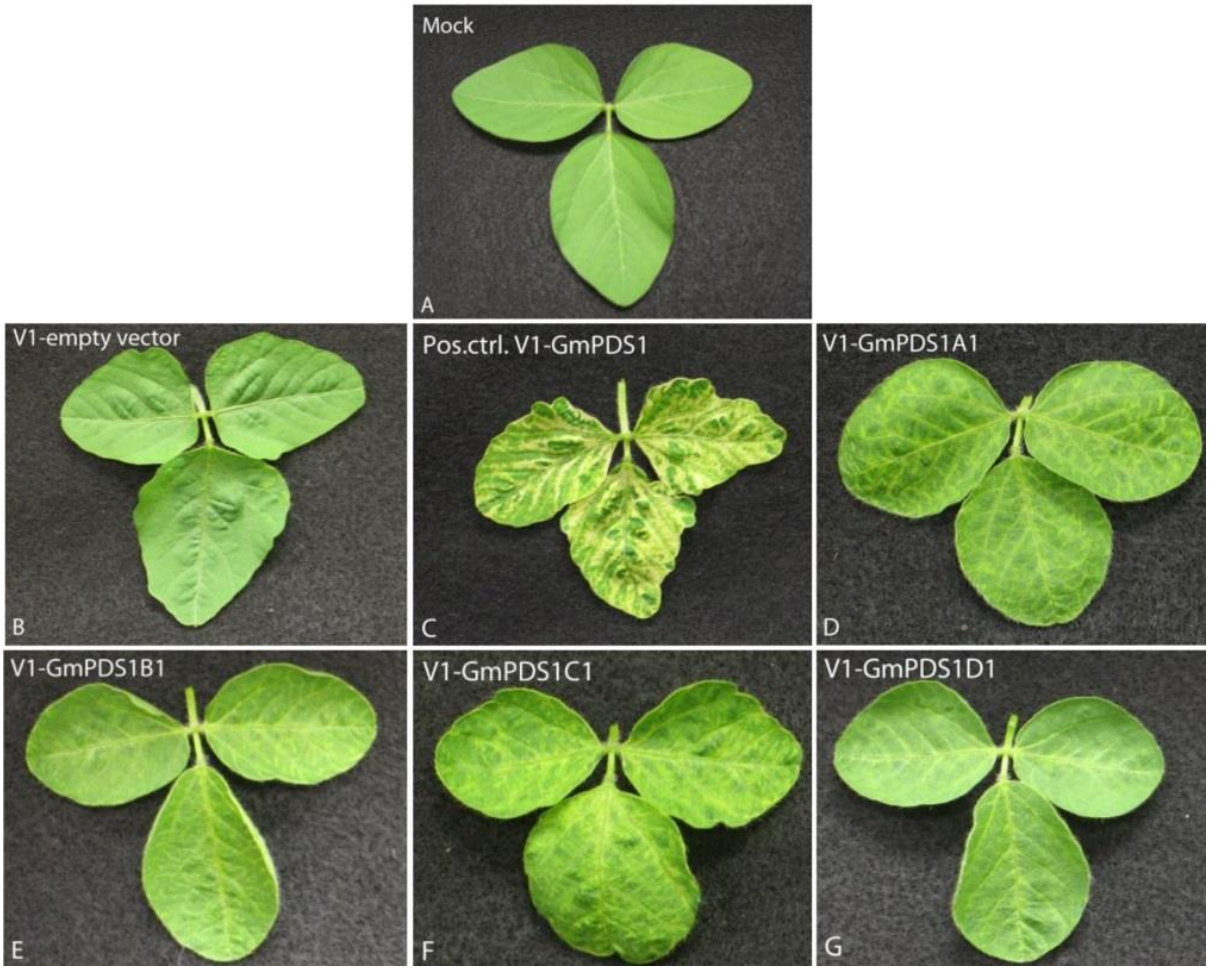

Figure 3.3: The impact of V1 mutant with its inserts in soybean plants grown in greenhouse. A: leaves of healthy control soybean plants. B and C: systemic symptoms of soybean plants infected with V1 construct either with or without soybean-derived insert, respectively. D-G: soybean systemic infected leaves with the four mutants containing 96 nt-long of soybean origin. BPMV-IA-RNA1M was used as the RNA1 clone for all inoculations. Images were taken at 15 dpi.

The inserts, GmPDS1A1, B1, C1 or D1, were cloned into the *Bam*HI site of V1 vector in antisense orientation (Fig. 3.1B). Recombinant V1 vectors, V1-GmPDS1A1 and V1-GmPDS1B1, were constructed carrying GmPDS1-derived fragments specific to two different regions within the 3' half of GmPDS1 ORF. However, V1-GmPDS1C1 and V1-GmPDS1D1 constructs were designed to target the 5' region of GmPDS1 ORF (Fig. 3.1C).

From the previous experiment, plants grown in the greenhouse and infected with pos.ctrl. V1-GmPDS1 construct showed extensive photobleaching with relatively mild virus symptoms. Therefore, all soybean plants used in the next experiments were reared in a greenhouse.

Leaves of soybean plants were inspected for the presence of photobleaching. Unlike the silencing of the GmPDS1 with sequences of 27nt-long, all the V1-based constructs containing GmPDS1A1, B1, C1 or D1 were able to initiate silencing, indicating silencing of GmPDS1. The photobleaching in all treatments was manifested by only white spots along the veins. While their ability to infect soybean plants and spread systemically, the V1-GmPDS1A1, B1, C1 or D1 construct induced weak photobleaching (Fig. 3.3D, E, F and G, respectively). In conclusion, despite targeted against four distinct regions of the GmPDS1, the 96nt-long inserts could probably not silence the GmPDS1 in soybean plants as efficient as those infected with the pos.ctrl V1-GmPDS1.

3.2 Nucleotide identity of the bottom part of the 5' UTR stem-loop C (SLC) of RNA2 is indispensable for BPMV infectivity

To facilitate the tracking of infection process, an RNA2 derivative that contained a GFP insert between MP and L-CP was used as the RNA2 surrogate. This mutant, referred to as RNA2-GFP or R2G as shown in fig. 3.4a, was shown previously to replicate to similar levels as that of wild-type RNA2 (Zhang *et al.*, 2010). Stem-loop C (SLC) is a 66nt-long, spanning from nt #400-466 of the 5' UTR of BPMV RNA2. The importance of the bottom part of SLC consisting of one C-U mismatch and six base pairs was examined (Fig. 3.4b). This portion of structure was disrupted by inserting four nts (aucc) after nt # 461, thus creating a *Bam*HI site in the corresponding cDNA (Fig. 3.4b, right panel).

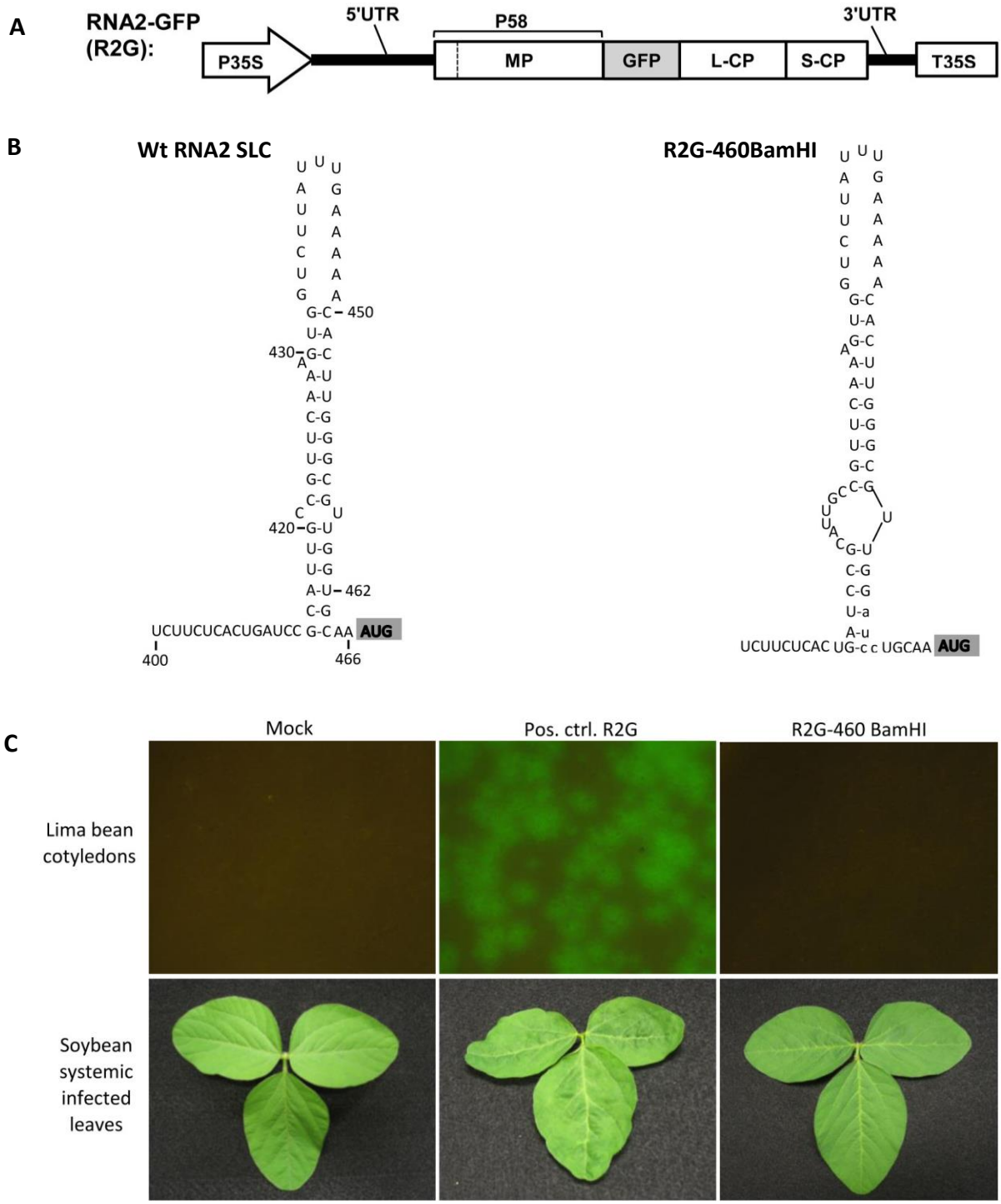

Fig. 3.4: The functionality of SLC depends on the nt identity of the bottom part of the stem. A: Schematic representation of RNA2 containing a GFP insert between MP and L-CP (R2G). B: Schematic representations of wt RNA2 SLC and R2G-460*Bam*HI. The altered nts in R2G-460*Bam*HI mutant are in lower-case letters. C: Infectivity of mutant construct assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants.

The resulting mutant, R2G-460*Bam*HI, completely abolished the BPMV infectivity (Fig. 3.4c, third row). It should be noted that MFold predicted that the upper two-thirds of SLC would remain essentially undisturbed in this mutant. In addition, the six base pair stem at the bottom of SLC also remained intact despite some changes in nt identity (Fig. 3.4b). If this predicted structural consequences is correct, then either the two bases (C-U) need to be faithfully preserved, or the identity of certain nts within this section of SLC is critically important. In summary, these results might support a critical role of SLC as a unique RNA structure required for RNA2 accumulation in the host cells of BPMV.

3.3 The 5' UTR of RNA2 tolerates large deletions and/or insertions

The 5' UTR of BPMV RNA2 is 466 nt long. As many as 137 nt of the 5' UTR could be deleted without compromising the infectivity of BPMV in its host plants lima bean and soybean (Lin *et al.*, 2013; Fig. 3.5, ΔSLA/B). This observation led to explain whether the same 5' UTR would accommodate large sized insertion of nonviral origins. A region of 47 nt long (nt #263-309, SLA) of BPMV RNA2 was deleted and replaced by a pair of restriction enzyme sites (*Bam*HI and *Acc*65I) to allow convenient insertion of foreign sequences. The resulting RNA2 mutant was designated V5UE (Fig. 3.5).

A 325 nt cDNA fragment of GmPDS1 was inserted into V5UE to test whether it can be tolerated by BPMV RNA2. Two BPMV RNA2-based constructs, V1-GmPDS1 and VM-GmPDS1, developed by Zhang *et al.*, (2010) containing the same 325 nt fragment of the GmPDS1 inserted within 3' UTR or in the middle (between MP and L-CP) of RNA2, respectively, were used to indicate the successful photobleaching due to the silencing of GmPDS1 mRNA (Fig. 3.5). In order to avoid undesired translational initiation within the GmPDS1 insert, all AUG start codons were removed.

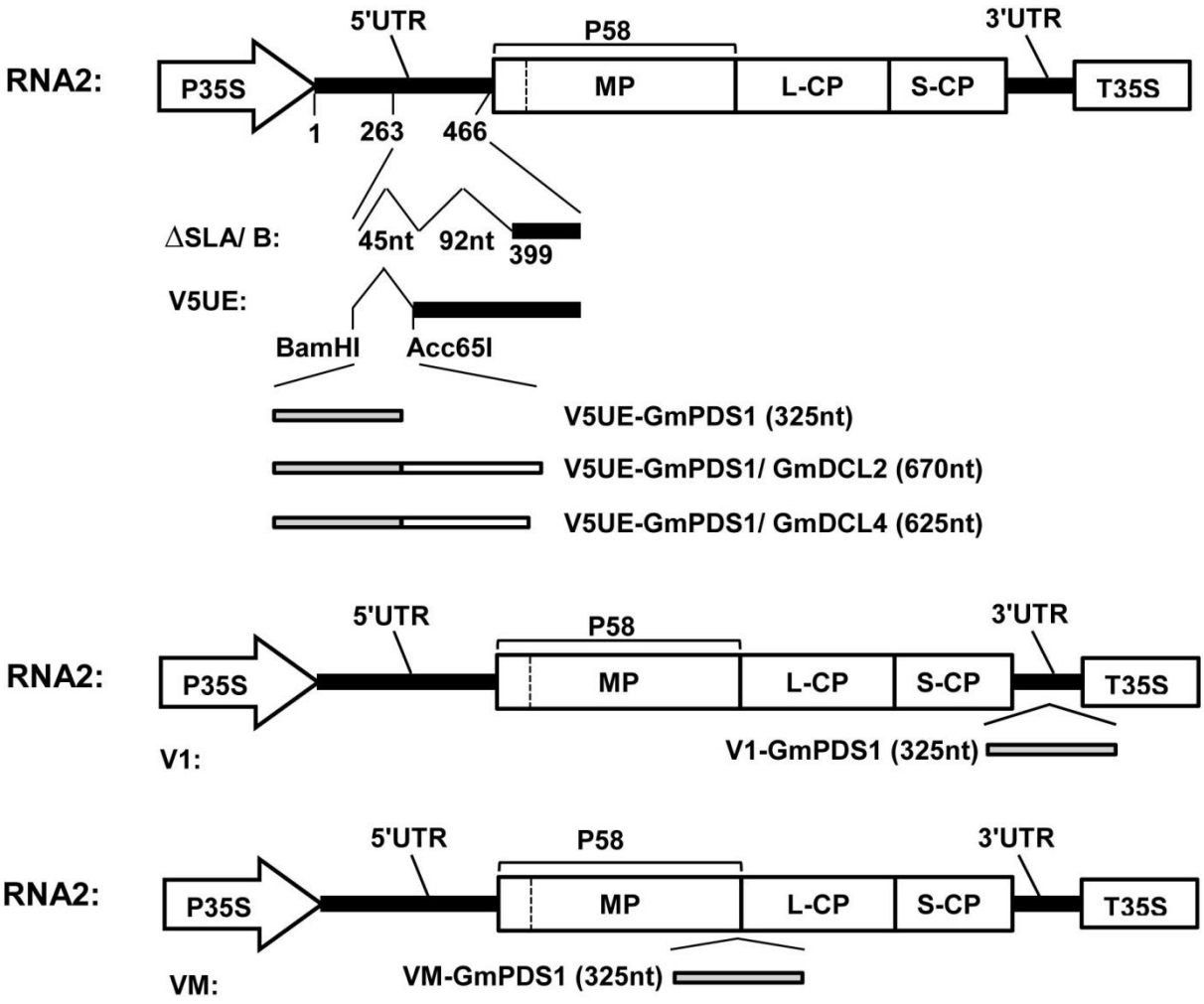

Figure 3.5: Schematic representations of BPMV RNA2-based constructs new V5UE, V1 and VM used in this study. The top diagram depicts the full length cDNA of RNA2 flanked by P35S and T35S. The 5' UTR is 466 nt long, with the two deletion mutants (ΔSLA/B) between nt #263 and 399 reported in an earlier study (Lin *et al.*, 2013). The V5UE mutant replaces nt #263-309 with *Bam*HI and *Acc*65I sites. The three insertion mutants derived from V5UE are depicted at the bottom. The V1 and VM insertion mutants that developed by the others (Zhang *et al.*, 2010) contain 325 nt-long fragment of PDS derived from soybean inserted within 3' UTR and in the middle (between MP and L-CP) of RNA2, respectively.

The inoculated soybean plants were reared in a greenhouse and daily inspected for symptom development. Fig. 3.6B shows that plants infected with the V5UE-empty vector developed mild symptoms with slightly deformed leaves. Surprisingly, comparing the plants infected with V1-GmPDS1 or VM-GmPDS1 mutant, it was found that plants infected with V5UE-GmPDS1

mutant showed extensive photobleaching on systemic infected leaves within 8dpi. Thus this observation indicated efficient multiplication and spread of the mutant, besides the retention of the GmPDS1 insert in the mutated viral RNA2 (Fig. 3.6C, D and E, respectively). The effective silencing of GmPDS1 by V5UE-GmPDS1 was proven by reverse transcriptase poly chain reaction (RT-PCR). Total RNA samples collected from systemic leaves at 15 dpi were subjected to RT-PCR for detection of GmPDS1 mRNA accumulation. As shown in Fig. 3.6F, top panel, the GmPDS1-specific RT-PCR products were tremendously at low levels in leaves infected with V1-GmPDS1, VM-PDS1 or V5UE-GmPDS1 (lanes 4, 5 and 6, respectively) compared with mock-inoculated control leaves (lane 2). The RT-PCR products were undetectable when reverse transcriptase (RT) was omitted, confirming them as mRNA-derived (Fig. 3.6F, bottom panel). Furthermore, 345 nt RT-PCR product of a soybean actin mRNA was used to ensure a similar amount of RNA used for each treatment (Fig. 3.6G). Finally, these results confirmed that V5UE-GmPDS1 induced as efficient silencing of GmPDS1as that of V1-GmPDS1 or VM-GmPDS1, suggesting that 325 nt GmPDS1-derived insert was probably stable in the construct.

In order to test the ability of V5UE mutant to permit nonviral inserts longer than 325 nt, two chimeric inserts of soybean origin, GmPDS1/GmDCL2 and GmPDS1/GmDCL4, were designed by adding cDNA fragments of GmDCL2 (345nts) and GmDCL4 (300nts), respectively, to the 325 nt-long GmPDS1 insert. The resulting two fragments with total lengths (670 and 625 nts) were inserted into V5UE mutant (Fig. 3.5). Plants infected with the V5UE-empty vector or V5UE-GmPDS1 showed mild symptoms or extensively photobleached systemic leaves, indicating systemic spread of virus mutants and efficient silencing of the GmPDS1 due to the V5UE-GmPDS1 infection (Fig. 3.7B and C, respectively). Plants infected with the V5UE-GmPDS1/GmDCL2

mutant, although showing symptoms similar to V5UE-empty vector-infected plant, developed only sporadic white spots on leaf margins or along the veins.

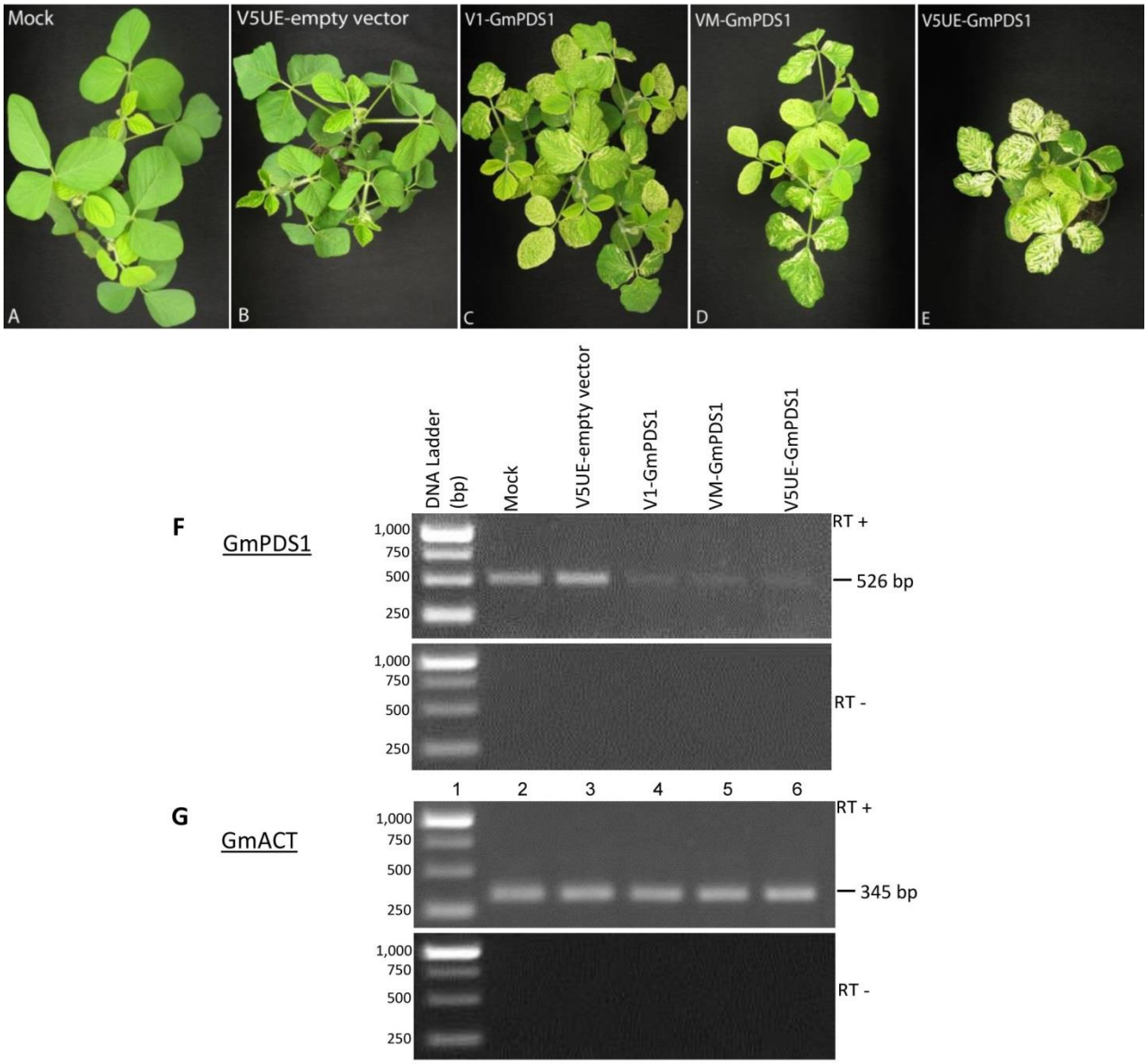

Figure 3.6: The V5UE and its GmPDS1 insertion in soybean plants. A: healthy soybean plants inoculated with only inoculation buffer. B-E: systemic symptoms of soybean plants infected with the four mutants. Images were taken at 15 dpi. BPMV-IA-RNA1M was used as the RNA1 clone for all inoculations. F-G: RT-PCR detection of mRNA levels of GmPDS1 and GmACT (soybean actin, control) in the systemically infected leaves. RT + and RT – in F and G denote RT-PCR reactions with or without reverse transcriptase, respectively.

This led to speculate that, while this construct was able to cause systemic infection in soybean, the GmPDS1 might not be stably maintained during the infection process (Fig. 3.7D). Notably, the V5UE-GmPDS1/GmDCL4, whose

insert was only 45 nt shorter than that of V5UE-GmPDS1-GmDCL2, induced large photobleaching in systemic leaves which are similar to those infected with V5UE-GmPDS1 (Fig. 3.7E). These results suggested that, unlike the GmPDS1a/GmDCL2 insert, the GmPDS1/GmDCL4 insert was probably relatively stable.

The GmPDS1-specific RT-PCR product in plants infected with V5UE-GmPDS1 or V5UE-GmPDS1/GmDCL4 was at visibly lower levels in leaves of plants infected with these two mutants (Fig. 3.7F, top panel, lanes 4 and 6).These data confirmed that V5UE-GmPDS1/DCL4 induced GmPDS1 silencing consistent with that caused by V5UE-GmPDS1, further suggesting that the GmPDS1/GmDCL4 (625 nts) might be stably maintained in the V5UE-GmPDS1/GmDCL4 mutant. Fig. 3.7F, bottom panel, displays the RT was excluded. RT-PCR product of GmACT indicated similar amount of RNA used for each treatment (Fig. 3.7G, top panel).

The DCL2-specific RT-PCR product was at similar level in all infected plants, including those infected with V5UE-GmPDS1/GmDCL2 (Fig. 3.7H, lane 5). This is consistent with symptoms of infected plants and RT-PCR results of GmPDS1, which both suggested the instability of the GmPDS1/GmDCL2 insert. Likewise, the GmDCL4-specific RT-PCR product was not reduced by infection with the V5UE-GmPDS1/GmDCL4 mutant (Fig. 3.7I, lane 6). This led to ask whether the DCL4 portion of the GmPDS1/GmDCL4 insert was maintained throughout the infection process.

To determine whether the inserts were lost, an RT-PCR was used to amplify the section of BPMV RNA2 genome spanning from nt #68 to #772 that encompasses all inserts. Indeed, the size of fragment (692 bp) amplified from V5UE-empty vector-infected plants matched the predicted size (Fig. 3.7K, lane 3). The size of fragment derived from V5UE-GmPDS1-infected plants was,

likewise, approximately 1,000 bp, closely matching the expected size of 1,011 bp (Fig. 3.7K, lane 4). Matching the expected size of 1,311 bp, the fragment derived from V5UE-GmPDS1/GmDCL4-infected plants was about 1,300 bp (Fig. 3.7K, lane 6). Examination of BPMV RNA1-specific RT-PCR fragment revealed an increase of RNA1 levels in plants with reduced GmPDS1 levels (Fig. 3.7J). These results revealed that the 5' UTR of BPMV RNA2 is able to accommodate nonviral inserts of up to 625 nt without affecting the virus infectivity.

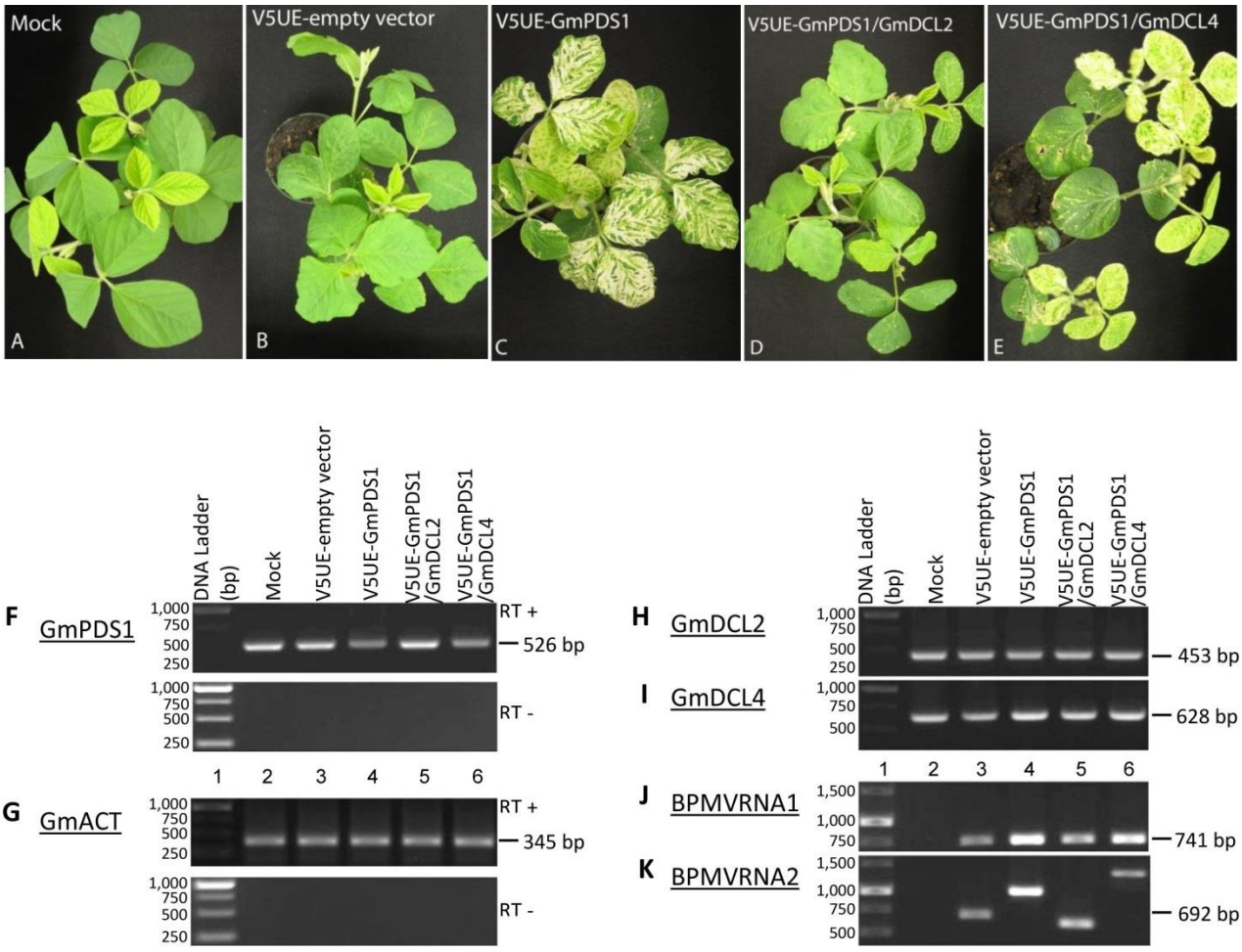

Figure 3.7: The V5UE with the three inserts of soybean origin in soybean plants. A: symptomless soybean plants. B-E: systemic symptoms of soybean plants infected with the four mutants. Plants were photographed at 15 dpi. BPMV-IA-RNA1M was used as the RNA1 clone for all inoculations. F-K: RT-PCR detection of mRNA levels of GmPDS1, GmACT (control), GmDCL2, GmDCL4, as well as the levels of BPMV RNA1 and RNA2 in the systemically infected leaves.

Surprisingly, the fragment amplified from systemic leaves infected with the V5UE-GmPDS1/GmDCL2 mutant was largely smaller than even that of the V5UE-empty vector mutant, confirming the insert in this mutant was deleted during the infection process (Fig. 3.7K, lane 5). The small size of the fragment derived from V5UE-GmPDS1/GmDCL2-infected plants further suggested that a portion of BPMV RNA2 5' UTR was lost together with the insert without compromising the infectivity of BPMV. The amplified fragment was then subjected to sequence analysis to determine the exact length and sequence of the deletion. Indeed, the sequencing result confirmed additional losses of RNA2 5' UTR sequences on both 5' and 3' sites of the insert. Fig 3.8 shows nt sequence alignments of the wild-type (Wt) RNA2, the V5UE mutant and the newly isolated deletion mutant. It is worth noting that the V5UE mutant replaced nt #263-309 (47 nts) of RNA2 with *Bam*HI and *Acc*65I sites (in blue letters). Furthermore, a new *Eco*72I site (CACGTG, in blue underlined letters) was also introduced to easily screen mutant constructs. Note that the GmPDS1/GmDCL2 insert was incorporated between the *Bam*HI and *Acc*65I sites. As shown in fig. 3.8 (the bottom line of the alignment), the newly generated truncation completely removed the insert and 13 additional nts (nt #250-262) upstream the insertion site, as well as 52 nts (nt #310-361) downstream the insertion site. The 5' UTR of this new RNA2 deletion mutant was 112 nts shorter than that of the Wt RNA2 and yet supported BPMV infections.

Inspecting the junction of deletion led to allocate a pair of identical sequences of 14 nts (TATAGGACTTCGTG, underlined) that flanked the insertion site. The new deletion mutant removed exactly the sequences between the two repeats, plus one of the repeats (Fig. 3.8). Therefore, the deletion could be probably caused by homologous recombination process. These results extended the 5' boundary of dispensable sequence to nt #250. Combined with

the previous study reported the sequence spanning the region of 137 nt-long (nt #263-399) could be removed without compromising BPMV infectivity (Lin *et al.*, 2013), it can be concluded that up to 150 nt (nt #250-399) of the BPMV RNA2 5' UTR could be deleted. There is no report showing the toleration of deletion of this size by the 5' UTR of a viral genomic RNA segment.

It can be reported that BPMV RNA2 mutants with deletions of up to 150 nts or nonviral insertions of up to 625 nts within its 5' UTR remained infectious in its host plant soybean. A key question is that why BPMV variants with such deletions were not recovered from fields of BPMV-infected plants. It could be speculate that these sequences might be required for BPMV propagation in other alternative hosts. It is known that BPMV overwinters in certain perennial weeds and spreads to soybean crops by bean leaf beetles that feed on both BPMV-containing weeds and soybean (Bradshaw *et al.*, 2008). Therefore, despite its dispensability for virus replication in soybean, the region spanning from nt #250 to #399 could be needed for efficient virus multiplication and survival in weedy alternative hosts, particularly under certain environmental conditions (for example low temperature).

Finally, tolerance of nonviral insertions of up to 625 nts 5' UTR of BPMV RNA2 offers the opportunity of designing new BPMV-based virus-induced gene silencing (VIGS) vectors that use the 5' UTR site to accommodate host gene fragments. VIGS is a powerful reverse genetics technique that assesses plant gene functions by down-regulating the expression of the corresponding gene with a replicating virus that carries a portion of coding sequence of that gene (Ruiz *et al.*, 1998). Since the replicating virus, called a VIGS vector, is targeted by the plant RNA silencing machinery to produce siRNAs that silence viral gene expression, the host gene fragment inserted in the VIGS vector is also subjected to the same process to produce siRNAs that specifically target the corresponding gene.

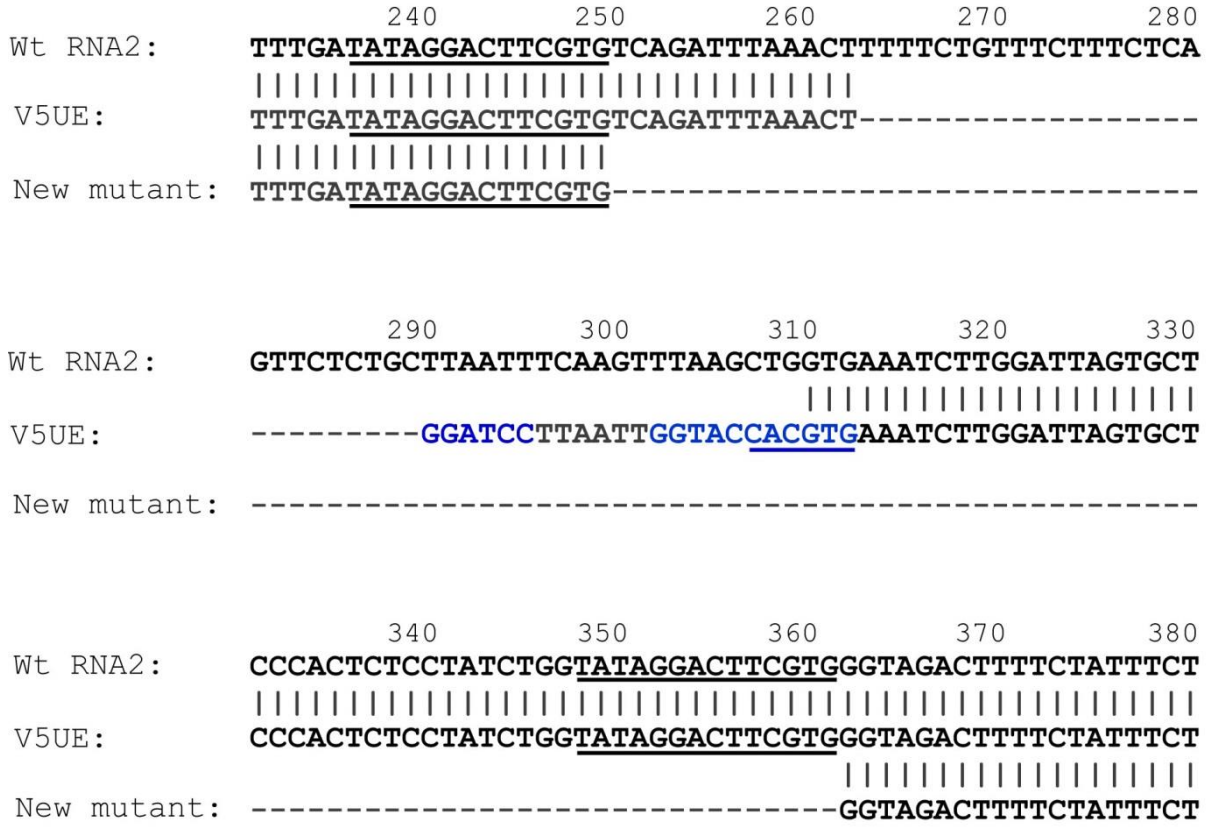

Figure 3.8: Alignment of the 5' UTR sequences of wild-type (Wt) BPMV RNA2, V5UE and the new mutant isolated from plants infected with V5UE-GmPDS1/GmDCL2. Only region spanning from nt #231 to #380 was shown. The 14-nt duplications in Wt RNA2 are underlined. The two new restriction enzyme (RE) sites (*Bam*HI and *Acc*65I) used for inserting nonviral sequences are highlighted in blue letters. They are separated by a 6-nt spacer (TTAATT) to ensure efficient digestion by both enzymes. Another new RE site (*Eco*72I) is highlighted in underlined blue letters.

BPMV has been used successfully as a VIGS vector in several previous reports (Zhang and Ghabrial, 2006; Zhang *et al.*, 2010; Diaz-Camino *et al.*, 2011; Pandey *et al.*, 2011). These vectors utilize sites in the middle (between MP and L-CP) or 3' UTR of BPMV RNA2 to accommodate fragments of host plant genes. Recent studies revealed that 5' termini of viral RNAs are potential candidates for siRNA production (Gracia-Ruiz *et al.*, 2010; Wang *et al.*, 2010). Therefore, a newly developed BPMV vector with insertion site within the 5' UTR of its RNA2 may prove to be a more rigorous VIGS vector. Indeed, data suggest that such a new vector is at least as efficient as previous vectors at

silencing the GmPDS1 expression. Furthermore, the 5' UTR of BPMV RNA2 reveals extraordinary level of flexibility and opens up new possibilities for improving BPMV-based VIGS vectors.

Conclusions

1. As early as 8 dpi, soybean plants developed virus symptoms when lima bean cotyledons that bombarded with BPMV RNA1 together with RNA2 cDNA constructs used as source of inoculum.
2. Greenhouse conditions were favorable for soybean plants to develop mild virus symptoms with extensive photobleaching.
3. V1-based vectors containing GmPDS1-derived inserts of 27 nt-long failed to induce photobleaching in soybean plants reared in growth chamber or greenhouse.
4. V1 VIGS vector derivatives with 96 nts targeting four different regions of GmPDS1 were able to develop sporadic photobleaching along the veins of soybean leaves.
5. The bottom portion of RNA2 SLC nt identity or C-U mismatch within that portion needed to be faithfully preserved for functional SLC that supports the BPMV RNA2 accumulation.
6. Replacing 47 nts (SLA, nt #263-309) of BPMV RNA2 5' UTR with restriction enzyme sites developed a new BPMV-based vector. The 5' UTR of BPMV RNA2 with deletion of up to 150 nts (nt #250-399) or nonviral insertions of up to 625 nts did not compromise the infectivity of BPMV in its host plant soybean.

Recommendations

1. Testing the possibility of the new mutant with up to 150 nts deletion (nt #250-399) to replicate and survive in weedy alternative hosts.

2. Determination the ability of the new deletion mutant to silence genes in its host plants by accommodating larger inserts.
3. Similar approach can be applied to other (+) RNA plant viruses with the aim of either exploring the indispensable sequences within the genome of virus or developing novel VIGS vectors.

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الخلاصة

تعد النواقل المشتقة من الفيروسات النباتية وسيلة قيمة للتحليل الجيني للنباتات المضيفة لتلك الفيروسات، ولها خواص مهمة في دراسة وظائف الجينات. ينتمي فيروس تبرقش قرنات الفاصوليا (*Bean pod mottle virus*, BPMV) والذي يمتلك جزيئتين موجبتين ومنفردتي السلسلة من الحامض النووي الرايبوزي (positive sense RNA) الى عائلة *Secoviridae*. ادى غرس قطع صغيرة نسبياً (96 قاعدة) من الدنا المتمم (cDNA) والمشتق من عدة مناطق من جين (Phytoene desaturase 1, PDS1) لفول الصويا (*Glycine max* L.) Merr ضمن (3' UTR) للقطعة الثانية من رنا الفيروس (RNA2) الى استحداث قصر ضوئي ضعيف (weak photobleaching) والذي تمثل بظهور بقع بيضاء منتشرة على طول عروق اوراق فول الصويا. وظفت خورازمية MFold للتنبؤ بالتركيب الثانوي (Secondary structure) لقطعة طولها 66 قاعدة (محصورة بين قاعدة 400-466) من النهاية 5' UTR لقطعة الرنا الثانية (RNA2) لفيروس BPMV. وجد ان لهذه القطعة امكانية الالتفاف بشكل stem-loop (SL) والتي سميت SLC. وجد ان الجزء السفلي من هذا التركيب له اهمية اساسية لحيوية الفيروس، اذ ان التلاعب بهذا الجزء من التركيب ادى الى فقدان قدرة فيروسBPMV على الاصابة. ازيلت 47 قاعدة والمحصورة بين قاعدة 263-309 من منطقة 5' UTR لقطعة الرنا الثانية (RNA2) لنفس الفيروس والتي سميت (SLA). غرست قطع من اصل غير فايروسي (GmPDS1 او GmPDS1/GmDCL4) والتي بلغت اطوالها 325 و625 قاعدة على التوالي والتي تم تقبلها من قبل المنطقة المزالة، اذ ادت الى تثبيط التعبير الجيني لجيناتها المطابقة. من ناحية اخرى، عانى احد الطافرات (V5UE-GmPDS1/GmDCL2) من عملية اعادة ارتباط (Recombination) اثناء التضاعف داخل نبات فول الصويا وفقدان قطعة طولها 112 قاعدة (محصورة بين قاعدة 250-361).

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

أَلَمْ تَرَ أَنَّ ٱللَّهَ أَنزَلَ مِنَ ٱلسَّمَآءِ مَآءً فَأَخْرَجْنَا بِهِۦ ثَمَرَٰتٍ مُّخْتَلِفًا أَلْوَٰنُهَا ۚ وَمِنَ ٱلْجِبَالِ جُدَدٌۢ بِيضٌ وَحُمْرٌ مُّخْتَلِفٌ أَلْوَٰنُهَا وَغَرَابِيبُ سُودٌ ﴿٢٧﴾ وَمِنَ ٱلنَّاسِ وَٱلدَّوَآبِّ وَٱلْأَنْعَٰمِ مُخْتَلِفٌ أَلْوَٰنُهُۥ كَذَٰلِكَ ۗ إِنَّمَا يَخْشَى ٱللَّهَ مِنْ عِبَادِهِ ٱلْعُلَمَٰٓؤُا۟ ۗ إِنَّ ٱللَّهَ عَزِيزٌ غَفُورٌ ﴿٢٨﴾

سورة فاطر (الايتان ٢٧–٢٨)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

استعمال فيروس تبرقش قرنات الفاصوليا(BPMV) *Bean pod mottle virus* كناقل لتحفيز الكبت الجيني لأستهداف عدة جينات في نبات فول الصويا *Glycine max* (L.) Merr.

اطروحة

مقدمة الى مجلس كلية العلوم، جامعة النهرين

كجزء من متطلبات نيل درجة الدكتوراه في التقانة الاحيائية

من قبل

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جمادى الأولى
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